CELL CULTURE TECHNOLOGIES FOR THE PRODUCTION OF BIOACTIVE COMPOUNDS FROM *RHODIOLA IMBRICATA* EDGEW. OF TRANS-HIMALAYAN LADAKH REGION

Thesis submitted in fulfillment of the requirements for the degree of

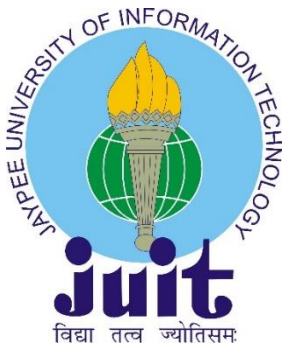
DOCTOR OF PHILOSOPHY

IN

BIOTECHNOLOGY

by

SAHIL KAPOOR



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DECLARATION

I hereby declare that the work reported in the Ph.D. thesis entitled **"Cell culture technologies for the production of bioactive compounds from *Rhodiola imbricata* Edgew. of trans-Himalayan Ladakh region"** submitted at **Jaypee University of Information Technology, Waknaghat, India,** is an authentic record of my work, carried out under the supervision of **Dr. Hemant Sood** and **Dr. Om Prakash Chaurasia.** I have not submitted this work elsewhere for any other degree or diploma. I am fully responsible for the contents of my Ph.D. thesis.

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CERTIFICATE

This is to certify that the work reported in the Ph.D. thesis entitled "**Cell culture technologies for the production of bioactive compounds from *Rhodiola imbricata* Edgew. of trans-Himalayan Ladakh region**" submitted by **Sahil Kapoor** (Enrollment no.: 136561) at **Jaypee University of Information Technology, Waknaghat, India,** is a bonafide record of his original work carried out under our supervision. This work has not been submitted elsewhere for any other degree or diploma.

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LIST OF ABBREVIATIONS

4CL	4-coumarate-CoA ligase
4-HPAA	4-Hydroxyphenylacetaldehyde
AA	Ascorbic acid
AAC	Ascorbic acid content
ABTS	2,2′-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)
AMPK	Adenosine monophosphate – activated kinase
ANOVA	Analysis of variance
ANS	Anthocyanidin reductase
AP2	APETLA 2
APRX	Alkaline metalloprotease
ATCC	American Type Culture Collection
BA/BAP	Benzyladenine or Benzylaminopurine
Bax	Bcl-2-associated X protein
Bcl-2	B-cell leukemia/lymphoma-2
bHLH	basic-helix-loop-helix
BHT	Butylated hydroxytoluene
C4H	Cinnamic acid 4-hydroxylase
CAB	Chlorophyll a/b-binding protein
CCA	Compact callus aggregate
CCC	Compact callus cluster
CDK-B	Cyclin-dependant kinase-B
CDPK	Calcium-dependent protein kinase
CHA	Chlorogenic acid
CHI	Chalcone isomerase
CHS	Chalcone synthase
CIF	Callus induction frequency
CLSI	Clinical & Laboratory Standards Institute
CNS	Central nervous system
COI1	CORONATINE INSENSITIVE 1
CRD	Completely randomized design
DAD	Diode array detector
DHAR	Dehydroascorbate reductase
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DRSA	DDPH free radical scavenging assay
DT	Doubling time
DW	Dry weight

eIF-2a	Eukaryotic initiation factor 2A
EOMT	Eugenol O-methyltransferase
ERF	Ethylene-responsive factor
F3H	Flavanone 3-hydroxylase
FLS-2	Flavonoid 3'-hydroxylase
FRAP	Ferric Reducing Antioxidant Power
FTC	Ferric thiocyanate
FW	Fresh weight
GC-MS	Gas chromatography–mass spectrometry
GI	Growth index
GME	GDP-D-mannose-3',5'-epimerase
GSH	Glutathione
HAT	Hydrogen atom transfer
HIF-1	Hypoxia-Inducible Factor-1
HMGR	3-hydroxy-3-methylglutaryl coenzyme A reductase
HPA	Hypothalamic–pituitary–adrenal
Hsp70	Heat shock proteins70
IAA	Indoleacetic acid
ICH	International Conference on Harmonisation
ISG	Interferon-stimulated gene
JA	Jasmonic acid
JAZ	JASMONATE ZIM-DOMAIN
JNK	c-Jun NH2-terminal kinases
LBD16	Lateral organ boundaries-domain 16
LEDs	Light emitting diodes
L-GalLDH	L-galactono-1,4-lactone dehydrogenase
LOD	Limit of deciation
LOQ	Limit of quantification
MAO	Monoamine oxidase
MAPK	Mitogen-activated protein kinase
MBC	Minimum bactericidal concentration
MDA	Malondialdehyde
MDA5	Melanoma differentiation-associated protein 5
MDAR	Monodehydroascorbate reductase
MdSnRK 1.1	Malus domestica Hexokinase 1 (HXK1) and Sucrose-Nonfermenting1 (SNF1)-related protein kinases 1
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant Staphylococcus aureus
MS	Murashige and Skoog
MYC2	Myelocytomatosis 2
NAA	Naphthaleneacetic acid
NIST	National Institute of Standards and Technology
NOX	NADPH oxidases
PAL	Phenylalanine ammonia lyase
PGRs	Plant growth regulators

PKC	Protein kinase-C
PKR	Protein kinase R
PPs	Protein phosphatases
PTFE	Polytetrafluoroethylene
QE	Quercetin equivalent
RE	Rutin equivalent
RGB	Red:Green:Blue
RIG1	Retinoic Acid Inducible Gene 1
ROS	Reactive oxygen species
RP-HPLC	Reverse Phase High Performance Liquid Chromatography
RSD	Relative standard deviation
SET	Single electron transfer
SGR	Specific growth rate
SOD	Superoxide dismutase
SPSS	Statistical package for the social sciences
STAT-3	Signal Transducer and Activator of Transcription 3
STRS	Streptomycin sulfate
TAC	Total antioxidant capacity
TBA	Thiobarbituric acid
Tdc	Tyrosine decarboxylase
TEAC	Trolox Equivalent Antioxidant Capacity
TF	Transcription factor
TFC	Total flavonoid content
TLC	Thin-layer chromatography
TPC	Total phenolic content
UFGT	UDP-Glc:flavonoid 3-O-glucosyltransferase
ZOI	Zone of inhibition

ABSTRACT

Rhodiola imbricata Edgew. is a traditional medicinal herb of Ladakh region. *R. imbricata* is a rich reservoir of phenolic compounds including phenylethanoids and phenylpropanoids. Salidroside and tyrosol are the major phenylethanoids of *Rhodiola* species, which are used in several herbal formulations for the treatment of stress- and age-related disorders. These bioactive compounds have aroused great industrial interest. The natural population of *R. imbricata* is rapidly decreasing and categorized as threatened in red data book. Therefore, the present study was focused on the establishment of cell culture system for countering extinction threats and to meet the escalating demands of industrially and medicinally valuable bioactive compounds of *R. imbricata.*

RP-HPLC method was validated for screening and selection of elite chemotypes with high content of phenylethanoids (salidroside and tyrosol). The RP-HPLC method was linear ($r^2 = 0.999$ in the range of 1 - 625 µg/ml), precise (RSD < 2.45 %) and accurate (recovery: 99.18 - 101.42 %; RSD < 1.92 %). Moreover, low detection (LOD: 0.10 - 0.15 µg/ml) and quantification limit (LOQ: 0.33 - 0.49 µg/ml) were suitable for the screening of phenylethanoids in *R. imbricata*. The RP-HPLC analysis for the first time demonstrated high variability in content of major phenylethanoids in different accessions of *R. imbricata*. KA4, CA1 and PA5 were identified as elite chemotypes of *R. imbricata* with high concentrations of major bioactive phenylethanoids.

The competence of cell culture system of *R. imbricata* has been explored for alternative synthesis of pharmaceutically important bioactive metabolites (phenolic compounds and ascorbic acid) to meet their increasing industrial demands. This study for the first-time showed the production of salidroside, phenolics, flavonoids and ascorbic acid in cell cultures obtained from elite chemotypes of *R. imbricata.* The maximum salidroside content (3.37 ± 0.03 mg/g DW) and biomass accumulation (8.43 ± 0.01 g/l DW) was observed in compact callus aggregates suspension cultures established in full strength MS medium fortified with 3 mg/l of BA and NAA, after 6 days of culture. The salidroside production was found to be growth-associated in cell cultures of *R. imbricata*. Callus and compact callus aggregates suspension cultures showed 1.23-fold and 1.52-fold higher content of salidroside, as compared

to the parent plant, respectively. GC-MS analysis demonstrated the presence of 71 compounds with pronounced biological activities in callus extracts of *R. imbricata*.

Since there is no information regarding the impact of light quality and jasmonic acid treatment on synthesis of bioactive compounds (Phenolics and ascorbic acid) in cell culture system of *R. imbricata*, therefore, this study explored the effect of different light conditions (blue, red, RGB, green and white) and different concentrations of jasmonic acid on synthesis of bioactive compounds and bioactivities in cell cultures of *R. imbricata*. The results demonstrated that blue light increased the levels of salidroside (1.45-fold higher than parent plant), phenolics (1.58-fold higher than control) and flavonoids (2.62-fold higher than control), whereas, red light enhanced the biomass accumulation (1.10-fold higher than control) in callus cultures. The CCA suspension cultures treated with 100 µM JA showed increased levels of salidroside (2.37-fold higher than parent plant), ascorbic acid (1.35-fold higher than control), phenolics (1.50-fold higher than control) and flavonoids (1.68-fold higher than control) along with the pronounced improvement in antioxidant and antimicrobial activities after 4 days of culture. Therefore, these findings open new possibilities for enhancing the production of industrially valuable bioactive metabolites in cell culture system of *R. imbricata*.

Hence, this study for the first time identified the elite chemotypes of *R. imbricata* with high content of major bioactive constituents by validated RP-HPLC method. In the present study, we have successfully established the cell culture system from elite chemotypes of *R. imbricata* for improved synthesis of bioactive molecules that bears immense industrial potential. The study has provided a first-time insight into the marked effects of light quality and jasmonic acid towards the enhanced production of bioactive compounds with the concomitant enhancement in antimicrobial and antioxidant activities in cell cultures of *R. imbricata*. These findings would be highly beneficial in realizing the escalating industrial demands of bioactive compounds from eco-friendly cell culture system of *R. imbricata*.

CHAPTER 1

INTRODUCTION

Rhodiola imbricata Edgew., locally known as (Shrolo), is an important medicinal plant of the Crassulaceae family. It is naturally distributed in cold desert regions of India, Pakistan, Nepal and China [**1**]. In India, the plant dwells along the stony terrains of high-elevation passes (Penzila, Changla, and Khardungla) in Ladakh (**Figure 1.1**) [**2**]. *R. imbricata* is extensively used in Amchi and Tibetan system of medicine for treating several health maladies, including, cough, cold, and CNS disorders [**3,4**]. Pharmacological studies have revealed that *R. imbricata* preparations exert antiviral, radioprotective, immunomodulatory, anti-cancer, immunostimulatory, hepatoprotective and antioxidant properties [**1,3, 5-10**]. The pharmacological properties of *R. imbricata* have been attributed to phenolic compounds, including, salidroside and tyrosol (phenylethanoids), rosin, rosavin and rosarin (phenylpropanoids), phenolic acid, flavonoids and flavonols [**1,11**]**.** Salidroside and tyrosol (phenylethanoids) are chief bioactive metabolites of *Rhodiola*, which are used as an important constituent in several herbal formulations, including ADAPT-232 and SHR-5 for the treatment of stress- and age-associated disorders [**12,13**]. SThese phenylethanoids derivatives possesses numerous pharmacological properties, including immunostimulatory, antiviral, anti-aging and antioxidative properties [**14,15**]. These compounds are arousing immense industrial and scientific interest [**16**].

Figure 1.1: *Rhodiola imbricata* plant growing at Khardungla pass of trans-Himalayan Ladakh region

R. imbricata is an exceptional source of essential amino acids, semi-volatile compounds, dietary mineral elements, tocopherols and vitamin B complex [**17-19**]. *R. imbricata* is substantially used in numerous herbal and pharmaceutical formulations [**20**]. The phytochemical extracts of *Rhodiola* species are widely used throughout the United States, Asia and Europe as nutraceutical and dietary supplement to improve resistance to stress, and work capacity [**21**]. The adaptogenic and tonic properties of *Rhodiola* species have been broadly used in Tibetan and Chinese traditional medicine [**22**]. A recent upsurge in worldwide demand of salidroside and this herb has resulted in extinction of several species of *Rhodiola* [**23**].

Earlier, *R. imbricata* roots and rhizomes were used for medicinal purposes by laypeople, but recently, commercial demand of *R. imbricata* have increased dramatically [**24**]. However, the natural resources of *R. imbricata* have decreased remarkably, chiefly owing to habitat destruction and overexploitation for medicine [**24, 25**]. Field cultivated *R. imbricata* plants have very low potency and are not considered to be useful for medicinal applications [**26**]. *R. imbricata* is categorized as threatened in red data book [**27**]. The organic synthesis of these compounds is not feasible due to high commercial investments [**28**]. Therefore, these constraints require the interventions of several biotechnological approaches for consistent synthesis of precious metabolites of *R. imbricata*. Plant cell cultures provide a sustainable and eco-friendly system for industrial production of high-value bioactive molecules [**29**]. This biotechnological technique has been instrumental in production of numerous commercially valuable drugs, including, vinblastine, paclitaxol and camptothecin etc. [**30**]. The bioactive product formation from plant cell cultures have several benefits over chemical synthesis or conventional cultivation methods, such as, production is consistent and independent of geographical, seasonal and climatic variations [**31**]. Plant cell cultures are valuable source of diverse phytochemicals with remarkable bioactivities, ranging from antioxidant, antimicrobial and anti-inflammatory activities [**32,33**]. Plant tissue cultures, especially callus cultures serve as useful system for production of various groups of bioactive metabolites [**34].** It has been widely used in the propagation of economically valuable traits and introduction of transgenes [**35**]. The callus cultures have many desirable features from biotechnological perspective, including easy cultivation, rapid proliferation and sustainable production of biologically active compounds [**36**]. Plant cell aggregates have been implicated in modulating cellular metabolism in suspension culture [**37**]. Cells within larger aggregates

are subjected to different microenvironments with respect to surface shear forces, nutrient availability and intercellular signalling [**37**]. The compact cell aggregates effectively influence the carbon flux and improve bioactive metabolites formation [**38**]. The compact callus aggregate (CCA) cultures show some degree of differentiation and limited oxygen diffusion that stimulates the biosynthesis and accrual of bioactive metabolites [**39**]. The cell aggregate suspension culture is widely utilized for synthesis of diverse array of bioactive metabolites, such as, phenylethanoid glycoside [**40**] and flavonoids [**41**]. However, till date, no study has been done to investigate the biosynthetic and bioactive potential of *R. imbricata* cell culture system.

The initiation of plant tissue culture usually begins with the selection of elite chemotypes that contains higher contents of the desired bioactive compounds [**31**]. The identification of superior chemotypes is of considerable significance for strain improvement, obtaining high producing cell lines, and to meet the stringent requirements of phyto-pharmaceutical industries [**42].** The selection of elite chemotypes is also essential due to high chemotypic variations discovered in different genotypes and ecotypes [**43**]. The superior chemotypes provide an important bio-resource for understanding the biosynthetic pathway of commercially important bioactive metabolites. Several researchers have established *in-vitro* cultures using elite accessions of different plant species [**44**]. However, there is no reports on the screening and selection of elite chemotypes of *R. imbricata*.

Plant cell culture systems offer a potential platform for sustainable production of secondary metabolites. However, they are generally restrained by inconsistent yield of bioactive metabolites **[45].** Elicitation is extensively utilized to boost the biosynthesis of bioactive molecules in *in-vitro* cultures [**30**]. Elicitation triggers various inducible defense mechanisms in plants [**46**]. Several studies have shown that the abiotic and biotic elicitors induce bioactive metabolites production via stimulating a metabolic network [**46**]. Light is regarded as a powerful abiotic elicitor of secondary metabolism in *in-vitro* cultures [**47**]. The diverse classes of plant photoreceptors (cryptochromes, phytochromes, phototropins and zeitlupes) sense distinct spectrum of light and regulates various physiological responses (photomorphogenic responses, secondary metabolism, growth and development) through specific signaling networks [**48**, **49**]. LEDs have evolved as encouraging lighting source for *in-vitro* systems [**50**]. LEDs are now extensively used as an efficient source of lighting in plant tissue culture experiments as it offers several advantages over conventional light

sources, such as, high luminous efficacy, high fluence rate, high wavelength specificity, low heat emissions, less energy consumption, compact, and economical [**51,52**]. LEDs offers a great promise to boost the biomass yield and bioactive metabolites formation in *in-vitro* cultures [**53**]. Different spectral lights (blue, green, white and red) have been used to increase the bioactivities and secondary product formation in *in-vitro* cultures of *Prunella vulgaris* [**54**] and *Artemisia absinthium* [**55**]. However, detailed information regarding optimal lighting parameters is still lacking [**56**] and, until date, there is no information regarding impact of light quality on secondary metabolism in cell cultures of *R. imbricata.*

Jasmonates are a class of oxylipins that are implicated in plant development, reproduction and stress responses [**57**]. JA is a powerful elicitor that modulates the synthesis of bioactive molecules in *in-vitro* cultures by triggering secondary metabolism [**58**]. JA also stimulates transcriptional networks comprising of COI1, MYC2, JAZ repressor proteins and AP2/ERF transcription factors that triggers secondary metabolism [**59**]. JA induces the accumulation of phenylpropanoids in cell culture of *Hypericum* species [**60**] and plumbagin in hairy roots of *Plumbago indica* [**61]**. However, as yet, no information exists on influence of JA in increasing the synthesis of bioactive molecules in cell cultures of *R. imbricata* and constitutes a domain of great commercial interest.

R. imbricata is a rich reservoir of bioactive compounds with high industrial and economical value. However, the natural population of *R. imbricata* is rapidly decreasing and it is considered as threatened in the red data book. Moreover, due to the extreme environmental constrains in high-altitude regions, the availability of plant material is not very secured. Hence, the development of alternate techniques and technologies are of foremost importance to meet the sustainable demand of bioactive compounds throughout the year, irrespective of any environmental constrains. Till today, other species of *Rhodiola* have been utilized by industries, but the exploration and sustainable availability of *R. imbricata* will definitely contribute to meet the rising demands of industries. Plant cell culture technologies provides a promising alternative platform for sustainable and improved accrual of bioactive molecules. However, there has been no study so far that accomplishes the establishment of cell culture system from elite chemotypes of *R. imbricata.* Moreover, the elicitation of bioactive compounds has not been investigated in cell cultures of *R. imbricata.*

Thus, the present study was focused on selection of elite chemotypes of *R. imbricata* and optimization of cell culture and elicitation conditions for obtaining cell line with improved production of biologically active compounds. RP-HPLC method was validated for screening and selection of superior chemotypes of *R. imbricata* with high content of major phenylethanoids. The culture media components *viz.* plant growth media, carbon source, sucrose concentration and PGRs concentration, and explant type were optimized to improve the biomass, accumulation of industrially important bioactive compounds (salidroside, tyrosol, phenolics, flavonoids and ascorbic acid) and biological activities (antioxidant and antimicrobial) in cell culture system of *R. imbricata*. GC-MS analysis was performed to ascertain the phytochemical composition and bioactive potential of callus cultures of *R. imbricata*. The potential of light quality and jasmonic acid was evaluated for enhancing synthesis of bioactive metabolites and bioactivities in callus and cell cultures of *R. imbricata*, respectively.

Therefore, considering the commercial, traditional and medicinal value of rare trans-Himalayan herb: *R. imbricata*, the present study was ventured with the following objectives:

- **Objective 1:** Screening and Selection of elite chemotypes of *R. imbricata* with high phenylethanoids (salidroside and tyrosol) content.
- **Objective 2:** Optimization of cell culture system for the production of bioactive compounds from elite chemotypes of *R. imbricata*.
- **Objective 3:** Elicitation of bioactive compounds in cell cultures of *R. imbricata*.
- **Objective 4:** Discerning the bioactivities (antioxidant and antimicrobial) in cell cultures of *R. imbricata*.

CHAPTER 2

REVIEW OF LITERATURE

Rhodiola is a relatively advanced group of the Crassulaceae family [**12].** The number of species in the *Rhodiola* genus varies between 60 [**62**] to 200 [**63**] and shows a high level of morphological diversity [**64].** *Rhodiola* originated on the Qinghai-Tibetan Plateau, and then dispersed to other sub-arctic regions of Asia, North America and Europe [**65].** The species diversity of *Rhodiola* is especially high in the Hengduan Mountains and the Qinghai-Tibetan Plateau in China [**64].** The uplifts of the Qinghai-Tibetan Plateau and reticulate evolution probably promoted species diversification in *Rhodiola* [**65].** *Rhodiola* could also be another lineage which originated in response to the Himalayan motion [**65].** Mayuzumi and Ohba [**62**] found that *R.* subgen. *Rhodiola* and both *R.* sect. *Chamaerhodiola* and *R.* sect. *Rhodiola* are polyphyletic in origin. Recent molecular analysis of Asian Crassulaceae demonstrated that the genus *Rhodiola* is well separated from *Sedum* [**62**].

The first written reference of *Rhodiola* medicinal use dates back to AD 77, when Dioscorides wrote of *rodia riza* in De Materia Medica [**66].** *Rhodiola* species, such as, *Rhodiola alterna, Rhodiola kirilowii, Rhodiola sachalinensis, Rhodiola crenulata, Rhodiola rosea and others,* is extensively utilized in traditional medicine as tonic and adaptogen [**16].** It is also used to treat anaemia, acute mountain sickness, gastrointenstinal ailments, depression, fatigue, infections, impotence and central nervous system disorders **[66, 67].** *Rhodiola* has a multi-targeted effect at the transcriptional level and it also affects several signaling pathways associated with beneficial effects on neurological, psychological, metabolic and cardiovascular disorders [**14].** Several years ago, the first *Rhodiola* products were introduced as adaptogens to the western world [**68].** *Rhodiola* has gained worldwide popularity as dietary supplements and natural herbal product **[69].** The *Rhodiola* extracts are now widely used throughout the United States, Asia and Europe to enhance mental alertness and physical vigor [**69].** Over 46 companies worldwide use *Rhodiola* extracts in their products [**12].** Currently, *Rhodiola* is excessively and indiscriminately collected from their natural habitats for pharmaceutical purposes [**64**]. As a result, several species of *Rhodiola* are now at a high risk of extinction [**12].**

R. imbricata is a traditional medicinal herb of Ladakh region, which has been widely used in various herbal drug formulations for the treatment of stress- and age-related disorders. The

pharmacological properties have been largely ascribed to the phenolic compounds. Considering the vast bioactivities and medicinal importance of *R. imbricata*, it is pivotal to compile previous research on this extremely important medicinal herb, as it could offer useful insights into an area yet unexplored. Therefore, in this chapter, we have reviewed the present knowledge on phytochemistry, pharmacological properties and current status of tissue culture, elicitation strategies and bioactivities in cell culture system of *R. imbricata*.

2.1 *R. imbricata*

The genus *Rhodiola* belongs to the Crassulaceae family. The family is arranged in the order Saxifragales, class Magnoliopsida and phylum Magnoliophyta. Linnaeus founded *Rhodiola* genus to include a single species, *Rhodiola rosea*, but in year 1777, Scopoli reduced *Rhodiola* to a section of Sedum [**70**]. The word *Rhodiola* is originated from Greek word "rhodon", referring to rose-like fragrant odor of fleshy rootstalk and Latin word "iola" means diminutive. The *Rhodiola* species is distributed in Asia, North America, and Europe [**71**].

R. imbricata is a dioecious, succulent perennial medicinal herb with rose scented massive rootstock. *R. imbricata* was first identified at an altitude of 11000-13000 ft in the Himalayan region by M. Pakenham Edgeworth [**72**]. *R. imbricata* is naturally distributed in cold desert regions of India, Pakistan, Nepal and China [**1**]. In India, it grows along the rocky slopes of Changla, Penzila and Khardungla pass in Ladakh region (**Figure 2.1, Figure 2.2)** [**2**]. It is commonly referred as Shrolo in Ladakh. The flowering stems of *R. imbricata* are glabrous (10-30 cm). The leaves are densely arranged, lanceolate, alternate, approximately imbricate, sessile, flat, glabrous, fleshy, entire and acute with blunt tip and nearly entire margin (2 - 3 cm x 3 - 7 mm). Rhizome is thick, aromatic and subcylindrical (2 - 2.5 cm). Inflorescence is compact and 20- to 40-flowered (1 - 1.5 x 2.5 - 3 cm), pedicel is glabrous (3 - 5 mm), calyx lobes are linear to subulate (3 - 4 mm) and petals are angular-oblanceolate and shorter than stamens (5 - 6.5 mm). Stamens are linear and obtuse, filament is 5.5 – 8 mm and anthers are dark purplish. Nectar scale is widely oblong and dark red (0.7 - 1.2 mm). Carpels are 3 – 5 mm each with 8 – 10 ovules and tapering into indistinct style. Fruits are 4-5 and 8 -12 mm and seeds are 1.5 mm and ellipsoid. The flowering and fruiting season is generally from July to September [**72, 73**]. *R. imbricata* is propagated through rootstocks cuttings and seeds. Under field conditions, 65 % of seed germination rate has been attained, while in rootstocks plantations, 86 % survival rate has been achieved [**74**].

R. imbricata is widely used in Amchi and Tibetan traditional medicine for treating several health maladies, including cough, cold and CNS disorders [**4**]. In Ladakh, a local dish "Tantur" is made from tender leaves of *R. imbricata* [**4**]. *R. imbricata* is known as dhodlli by the local communities of Gujjar and Bakerwal in Jammu and Kashmir, and its roots are generally chewed to treat fatigue [**75**]. Its roots are also used in numerous pharmaceutical, nutraceutical and herbal preparations [**20**].

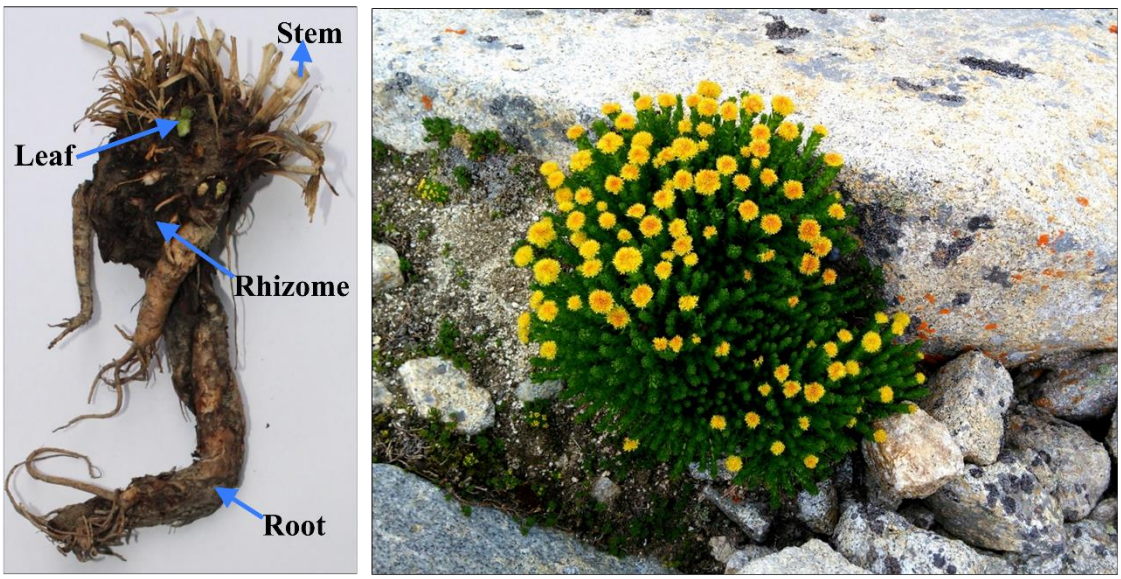


Figure 2.1: *Rhodiola imbricata* growing at Changla pass (17,586 ft).

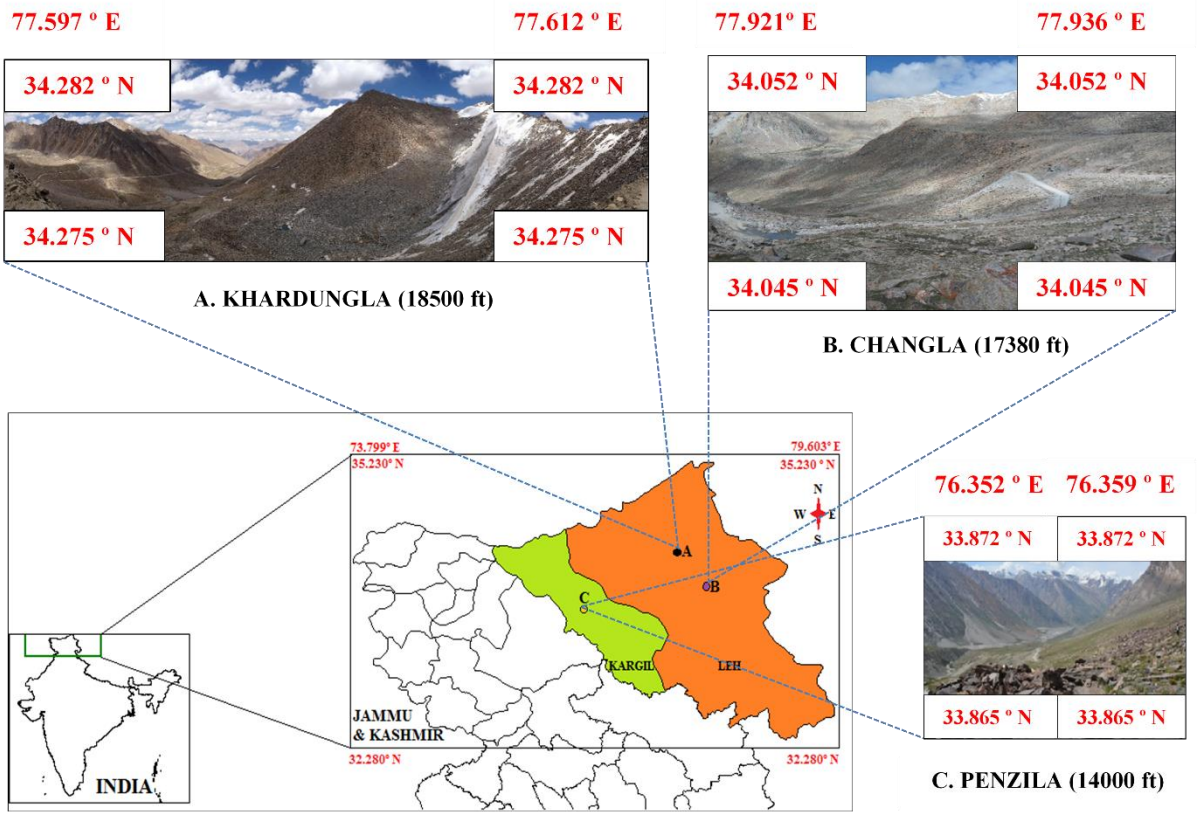


Figure 2.2: Geographical distribution of *Rhodiola imbricata* in trans-Himalayan region of Ladakh.

2.2 Phytochemistry of *R. imbricata*

Phytochemical research on *Rhodiola* species began in 1960s in Scandinavia and USSR [**66],** which further led to the discovery of numerous bioactive phytochemicals in the roots and rhizome of *Rhodiola* species. These bioactive compounds include cyanoglycosides, monoterpenoids, triterpenes (daucosterol, beta-sitosterol) phenylethanoids (salidroside, tyrosol), flavonoids (rhodiolin, rhodionin, rhodiosin), phenylpropanoids (rosin, rosarin, rosavin, cinnamyl alcohol) and phenolic acids (chlorogenic, hydroxycinnamic, gallic) [**76, 66].** Over 140 phytochemicals belonging to several distinct classes have been identified from *Rhodiola rosea* [**77**]. The essential oil of *Rhodiola rosea* is reported to contain 86 distinct phytochemicals and geraniol has been identified as the chief compounds associated with the rose-like odour of the roots [**78**]. The phytochemical constituents in *Rhodiola* are species-dependent, whilst certain overlap in components has been reported [**77].**

R. imbricata contains diverse phenolic compounds, including gallic acid [**8**], p-tyrosol [**11**], salidroside [**79**], rosin [**79**], rosavin [**79**], rosarin [**79**], cinnamyl alcohol [**79**], total flavonoids [**80, 1**], total phenolics [**80, 1**], total phenolic acid [**1]** and total flavonol [**1]** (**Figure 2.3**). It also possesses diverse array of health-promoting constituents like essential amino acid (histidine (1.43 ± 0.01 mg/g) and lysine (1.33 ± 0.02 mg/g)), fatty acid (capric acid (16.2 ± 0.41 mg/g), linoleic acid (12.2 ± 0.31 mg/g) and oleic acid (10 ± 0.25 mg/g)), dietary mineral elements (calcium (11.34 ± 0.33 g/kg) and potassium (2.14 ± 0.07 g/kg) fat-soluble vitamin (vitamin-E) and water-soluble vitamin (vitamin- B3, B5 and B6) in roots (**Figure 2.4**) [**17,18**].

Several volatile and semi-volatile phytochemicals have been identified in roots of this plant (**Figure 2.5**) [**19**]. Choudhary et al. [**3**] have extracted and characterized four new compounds and other known phenolic glycosides from root extract of *R. imbricata* (**Figure 2.6**).

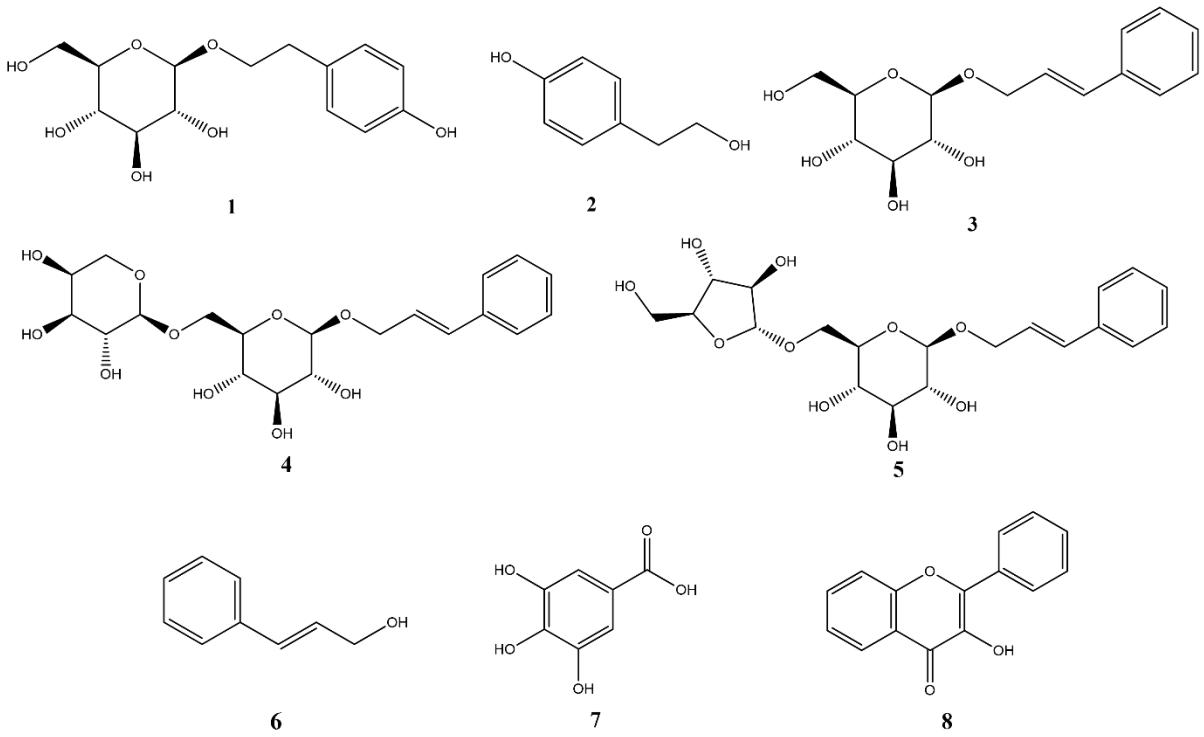


Figure 2.3: The diverse phenolic compounds of *Rhodiola imbricata* (**1**: Salidroside, **2**: Tyrosol, **3**: Rosin, **4**: Rosavin, **5**: Rosarin, **6**: Cinnamyl alcohol, **7**: Gallic acid, **8**: Flavonol).

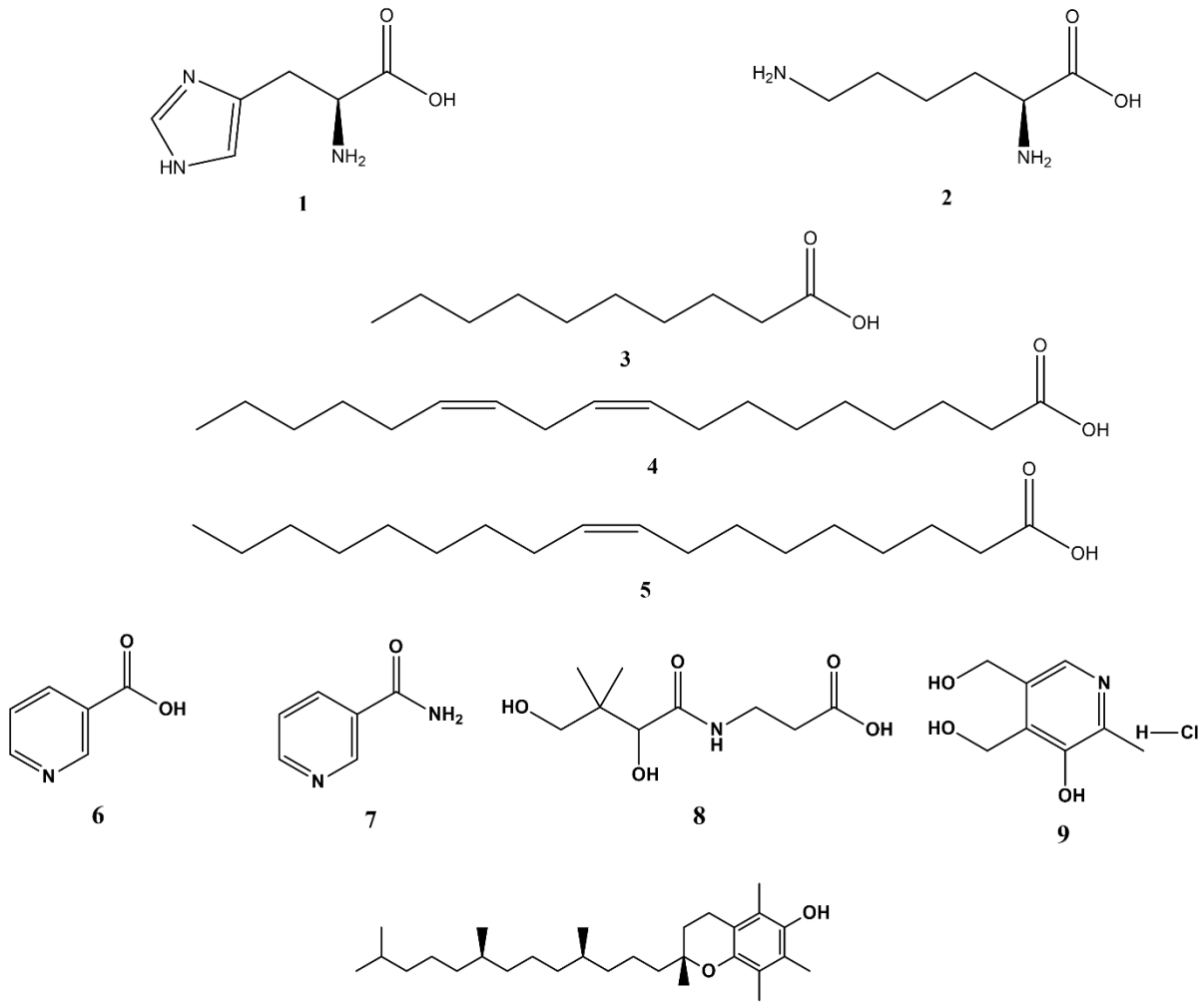


Figure 2.4: Essential amino acids ((**1**) Histidine and (**2**) Lysine), fatty acids ((**3**) Capric acid, (**4**) Linoleic acid and (**5**) Oleic acid), water-soluble vitamins ((**6**) Nicotinic acid (vitamin-B3); (**7**) Nicotinamide (vitamin-B3); (**8**) D-pantothenic acid (vitamin-B5); (**9**) Pyridoxine (vitamin-B6)) and fat-soluble vitamins ((**10**) D-a-tocopherol (vitamin-E)) identified in roots of *Rhodiola imbricata* [**17, 18**].

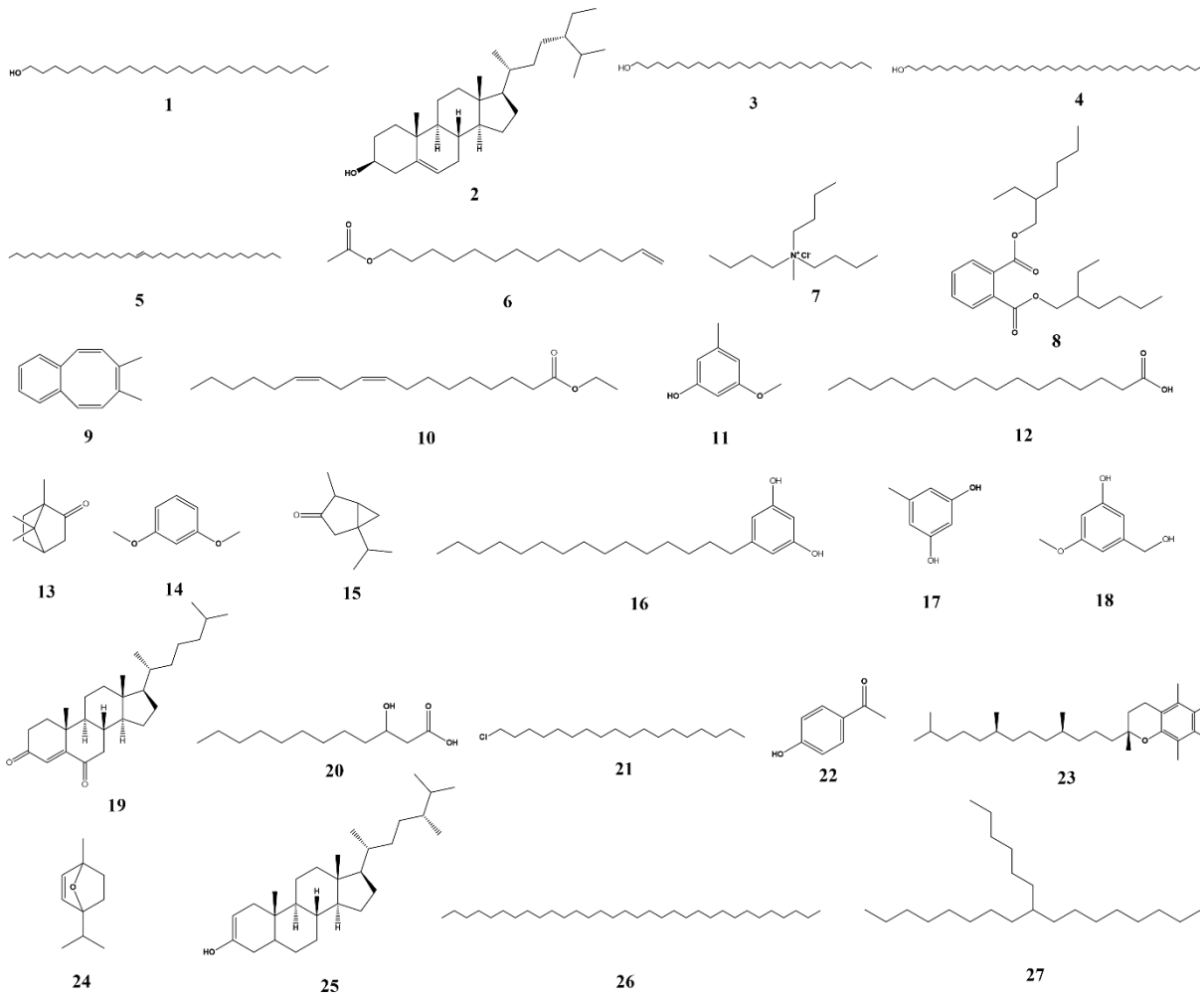


Figure 2.5: Major phytochemicals identified in different root extracts of *R. imbricata* by GC-MS. **1:** 1-pentacosanol; **2:** stigmast-5-en-3-ol, (3β,24S); **3:** 1-teracosanol; **4:** 1-henteracontanol; **5:** 1-pentatriacontene; **6:** 1-tetradecen-1-ol acetate; **7:** methyl tri-butyl ammonium chloride; **8:** bis(2-ethylhexyl) phthalate; **9:** 7,8-dimethylbenzocyclooctene; **10:** ethyl linoleate; **11:** 3-methoxy-5-methylphenol; **12:** hexadecanoic acid; **13:** camphor; **14:** 1,3-dimethoxybenzene; **15:** thujone; **16:** 1,3- benzenediol, 5-pentadecyl; **17:** 1,3-benzenediol, 5-methyl; **18:** benzenemethanol, 3-hydroxy, 5-methoxy; **19:** cholest-4-ene-3,6-dione; **20:** dodecanoic acid, 3-hydroxy; **21:** octadecane, 1-chloro; **22:** ethanone, 1-(4-hydroxyphenyl); **23:** a-tocopherol; **24:** ascaridole; **25:** campesterol; **26:** dotriacontane; **27:** heptadecane, 9-hexyl [**19**].

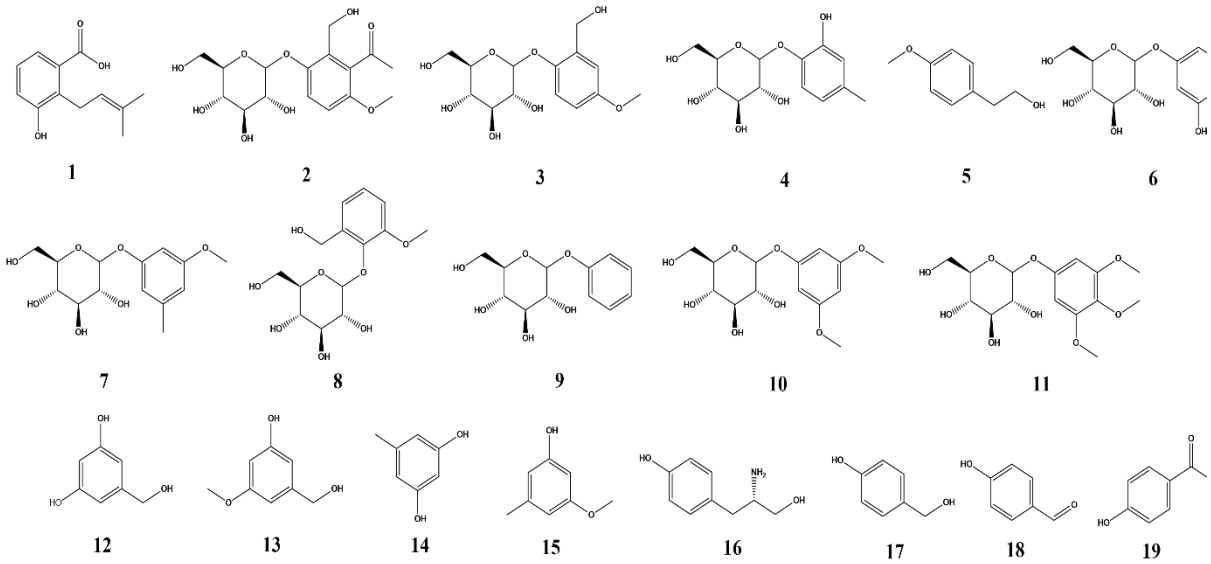


Figure 2.6: Phenolic compounds identified in ethyl acetate root extract of *R. imbricata*. **1:** 3-hydroxy-2-(3-methyl-2-buten-1-yl)- benzoic acid; **2:** 3-acetyl-2-(hydroxymethyl)-4-methoxyphenyl-*β*-D-glucopyranoside; **3:** 2-(hydroxymethyl)-4-methoxyphenyl-*β*-D-glucopyranoside; **4:** 2-hydroxy-4-methylphenyl-*β*-D-glucopyranoside; **5:** 4-methoxyphenethyl alcohol; **6:** 3-hydroxy-5-methylphenyl- *β*-D-glucopyranoside; **7:** 3-methoxy-5-methylphenyl-*β*-D-glucopyranoside; **8:** 6-hydroxymethyl-2-methoxyphenyl-*β*-D-glucopyranoside; **9:** phenyl-*β*-D-glucopyranoside; **10:** 3,5-dimethoxyphenyl-*β*-D-glucopyranoside; **11:** 3,4,5-trimethoxyphenyl-*β*-D-glucopyranoside; **12:** 3,5-dihydroxybenzyl alcohol; **13:** 3-hydroxy-5-methoxybenzyl alcohol; **14:** orcinol; **15:** *O*-methylorcinol; **16:** Tyrosol; **17:** *p*-hydroxybenzylalcohol; **18:** *p*-hydroxybenzaldehyde; **19:** *p*-hydroxyacetophenone [**3**].

2.3 Pharmacological properties of *R. imbricata*

Pharmacological research on *Rhodiola* species began in 1960s in Scandinavia and USSR [**66].** Between 2000 and 2015, Asian researchers have published numerous research articles on pharmacological properties of different *Rhodiola* species [**81].** *Rhodiola* preparations affects several physiological functions, including CNS activity and cardiac functions [**5**]. The pharmacological preparations of *Rhodiola* species have been reported to regulate energy balance, anxiety, depression, memory and learning through probable interactions with neuropeptide Y receptors [**77].** *Rhodiola* species have been reported to possess cytoprotective [**82]**, adaptogenic [**7**], anti-hypoxic [**83**], anti-fatigue [**84**], neuroprotective [**85**], hepatoprotective [**86**], stress-protective, anti-depressive and anxiolytic activity [**14**]. The anti-stress activity of *Rhodiola* is associated with regulation of HPA axis and Hsp70 [**14**],

JNK1 [**87**], DAF-16 [**87**] and beta-endorphine [**14**]. The pharmacological properties of *Rhodiola* are species-dependent, whilst certain overlap in bioactivities have been reported [**10].**

R. imbricata is one of the most important species of the genus *Rhodiola,* which is broadly utilized in modern and traditional medicine. It is traditionally used for treating fever, cough, cold and CNS disorders [**4**]. The pharmacological properties of *R. imbricata* are displayed in **Table.2.1**. The major pharmacological properties have been described below:

2.3.1 Cytoprotective and Radioprotective activity

Kanupriya et al. [88] reported that root extract of *R. imbricata* (250 µg/ml) have a marked cytoprotective activity against BHT (250 µM) in human macrophages. **Chawla et al. [79]** found that antioxidant capacity of *R. imbricata* contributed significantly towards its radioprotective efficacy. **Goel et al. [89]** revealed that administration of a single dose of aqueous root and rhizome extracts of *R. imbricata* in Swiss albino strain 'A' mice rendered 83% or more survival (at 30 days) against whole-body lethal gamma irradiation (10 Gy). **Arora et al. [4]** revealed that the intraperitoneal administration of hydro-alcoholic rhizome extract of *R. imbricata* in Swiss albino strain 'A' mice rendered 83.3% survival against total-body gamma-irradiation (10 Gy).

2.3.2 Antioxidant activity

Avasthi et al. [80] investigated the antioxidant activity of whole plant extract of *R. imbricata* by bioassay guided fractionation method. The results showed that the dichloromethane extract of *R imbricata* exhibited the highest DPPH free radical scavenging activity (IC_{50}: 133.41 ± 0.04 µg/ml) and the ethyl acetate extract of *R. imbricata* has the maximum ferric reducing antioxidant power (EC_1: 92.13 ± 0.14 µg/ml). The *R. imbricata* extract exert strong antioxidant capacity in FTC, TBA and DRSA assays [**3**]. **Senthilkumar et al. [8]** also reported that the methanol (IC_{50}: 62.80 µg/ml) and acetone (IC_{50}: 63.80 µg/ml) extracts of rhizome of *R. imbricata* showed maximum DPPH radical scavenging activity that was comparable to the reference standards BHT (IC_{50}: 45.56 µg/ml). **Tayade et al. [1]** reported that the methanolic root extract showed significantly high ABTS and DRSA. **Gupta et al. [11]** reported that blood MDA, GSH and SOD activity restricted significantly after administration of *R. imbricata* root extract.

2.3.3 Antiviral activity

Diwaker et al. [5] revealed that *R. imbricata* aqueous extract (50 or 100 µg/ml) induces RIG-I, MDA5 and ISG, that decreases the intracellular dengue virus load. Moreover, *R. imbricata* aqueous extract also upregulated phosphorylated eIF-2a, PKR and NF-kB in infected cells.

2.3.4 Wound healing and Adaptogenic activity

The *R. imbricata* rhizome extract heal experimental wounds in rats much faster in comparison to positive control [**90**]. *R. imbricata* root extract demonstrated high adaptogenic activity as cold-hypoxia-restrained rats showed faster recovery from hypothermia [**7**].

2.3.5 Immunostimulatory and Adjuvant activity

Mishra et al. [91] found that rhizome extract of *R. imbricata* stimulates the production of interleukin-6 and TNF- a in mononuclear cells and RAW 264.7 cell line. **Mishra et al. [92]** found that rhizome extract of *R. imbricata* has remarkable adjuvant activity against tetanus toxoid in rats.

2.3.6 Antiproliferative activity

Senthilkumar et al. [93] found that rhizome extracts of *R. imbricata* inhibits the spread of colon cancer cells. The antiproliferative activity of the acetone and methanol extracts may be due to the presence of phenolics in *R. imbricata* rhizome. **Mishra et al. [94]** observed that the proliferation of human erythroleukemic cell line was significantly decreased with *R. imbricata* rhizome aqueous extract.

2.3.7 Hepatoprotective activity

Senthilkumar et al. [8] found that *R. imbricata* extract protects hepatocytes against paracetamol induced hepatotoxicity in rats.

2.3.8 Antibacterial and Antifungal activity

The aqueous residue of *R. imbricata* whole plant extract exert strong antibacterial activity against *Escherichia coli* and *Serratia* sp., whereas the ethyl acetate extract exhibited

maximum antibacterial activity against *Acinetobacter* sp., *Staphylococcus aureus* and *Serratia* sp. **[95]**.

Avasthi et al. [95] found that extract of *R. imbricata* exhibited significant antifungal activity against *Fusarium moniliforme*, whereas the ethyl acetate and dichloromethane extract exhibited maximum antifungal activity against *Candida albicans*.

Table 2.1: Pharmacological properties of *Rhodiola imbricata*

S. No.	Pharmacological property	Reference
1	Adaptogenic activity	**[7]**
2	Adjuvant activity	**[91]**
3	Antibacterial activity	**[94]**
4	Anticancer activity	**[3]**
5	Antifungal activity	**[94]**
6	Antioxidant activity	**[80]**
7	Antiproliferative activity	**[92]**
8	Antiviral activity	**[5]**
9	Cytoprotective activity	**[87]**
10	Hepatoprotective activity	**[8]**
11	Immunomodulatory activity	**[96]**
12	Immunostimulatory activity	**[90]**
13	Radioprotective activity	**[79]**
14	Wound healing activity	**[89]**

2.4 Pharmacological properties of Salidroside and Tyrosol

2.4.1 Salidroside

Salidroside is a β-D-glucopyranoside of β-(p-hydroxyphenyl) ethanol [**97**]. Salidroside is considered to be the most important bioactive constituent of *Rhodiola* [**98**]. It is utilized as an adaptogen in traditional medicine [**99**]. Salidroside is a strong antioxidant and anticarcinogen [**100**]. It helps in preventing stress-mediated damages and neuro-endocrine disorders [**77**]. Salidroside exhibit protective action against oxidative damage [**101**]. It has been reported to

improve cognitive function, relieve myocardial ischemia, and prevent and treat mountain sickness [**102**]. Some of the major pharmacological properties of salidroside have been described below:

2.4.1.1 Neuroprotective activity

Salidroside protect neuron cells by inducing several antioxidant enzymes and also by downregulating Bax and upregulating Bcl-2 and Bcl-XL [**103**]. It also stimulates the expression of neuropeptide Y (NPY) and Hsp-72 in isolated human neuroglia cells **[104**].

2.4.1.2 Anti-inflammatory activity

Salidroside reduces inflammatory response by preventing STAT3 transfer into nucleus [**105**]. It acts as an anti- inflammatory agent by blocking MAPK and NF-kβ signaling pathways [**106**].

2.4.1.3 Anticancer activity

Salidroside decreased the viability of bladder cancer cells through the MMP-9 signaling pathways [**107**]. Salidroside significantly decreased the multiplication of A549 cells by reducing pp38 protein expression [**108**].

2.4.1.4 Hepatoprotective activity

Salidroside protects against cadmium-induced hepatotoxicity in rats via GJIC and MAPK pathways [**109**]. It also changes the distribution of CD4β lymphocytes in spleen by regulating CXCL-10 [**110**]. Salidroside attenuates fatty liver disease via AMPK-dependent TXNIP/NLRP3 pathway [**111**].

2.4.1.5 Cardioprotective activity

Salidroside exerts protective effect against myocardial ischemia–reperfusion injury via activation of AMPK/PGC-1α and AMPK/NF-κβ signaling cascades [**112**]. Salidroside protects cardiomyocytes against oxygen glucose deprivation via activating Akt signaling [**113**]. It also inhibits cardiomyocytes apoptosis by up-regulating HIF-1a expression and VEGF levels [**114**].

2.4.1.6 Anti-dengue virus activity

Salidroside exerts anti-dengue activity by modulating IRF-7, PKR, IRF-3, IFN-a, P-eIF2a and RIG-I. Moreover, the salidroside treatment increased NK cells and CD8+ T cells in human PBMCs, which is highly critical in limiting the dengue virus replication during early stages of infection [**115**].

2.4.2 Tyrosol

Tyrosol (4-(2-hydroxyethyl) phenol) is a phenylethanoid, which exerts numerous bioactivities, including antioxidant, anticancer, neuroprotective and antihyperglycemic activities [**116**]. Tyrosol inhibits GSH consumption to protect against oxidative stress [11**7]**. It also ameliorates hyperglycemia in streptozotocin induced diabetic rats [**118**]. Tyrosol significantly reduced lipoperoxidation in neural tissue of animal's subjected to global cerebral ischemia [**119**]. Tyrosol suppresses tyrosinase activity in B16F0 cells [**120**]. It also significantly increased superoxide dismutase activity during the differentiation of preadipocytes [**121**].

2.4.3 Rosavin and other phenolic compounds

Rosavin exhibited strong anti-depressant activity by enhancing the stimulating properties of L-DOPA and inhibiting monoamine oxidases [**122**]. Rosavin showed a stimulatory influence on CNS activity [**123**]. It also reveals strong antibacterial inhibitory activity against resistant strains of *Neisseria gonorrhoeae* [**123**]. Rosavin exhibits acetylcholinesterase (AChE) and xanthine oxidase (XOD) inhibitory activities [**124**]. It also showed antiangiogenic and antitumor activities by probably inhibiting the farnesyl protein transferase and cell-cell adhesion [**125**]. Other phenolic compounds, such as, Kaempferol from *Rhodiola rosea* displayed potent inhibitory activity against influenza virus [**12].** Gossypetin-7-O-l-rhamnopyranoside and rhodioflavonoside isolated from the stems of *Rhodiola rosea* demonstrated potent inhibitory activity against *Staphylococcus aureus* [**12]**. It also showed strong cytotoxic activity against prostate cancer [**12]** and acetylcholinesterase inhibitory activity [**12].** Rhodiosin from *Rhodiola rosea* demonstrated potent antioxidant activity against oxygen free radicals [**126**]. Gallic acid, kaempferol and *Rhodiola sachalinensis* displayed powerful DPPH free radical scavenging activity [**127**].

2.5 The biosynthesis of Salidroside and Tyrosol

Salidroside is biosynthesized from its aglycone precursor, tyrosol, by the enzymatic addition of glucose via a regio-specific uridine 5-O-diphospho-glucosyltransferase (UDP-glucosyltransferases) [**128**] or by β-d-glucosidases [**12**]. The salidroside and tyrosol biosynthesis has been displayed in **Figure 2.7**. There is an ambiguity related to the biosynthesis of salidroside and its aglycone precursor, tyrosol. Salidroside is believed to be produced through two distinct biosynthetic routes. According to some authors, tyrosol, aglycone precursor of salidroside, is synthesized from tyrosine through three distinct enzymes, namely, Tdc, MAO and 4-HPAA [**128**]. while others believe that tyrosol is biosynthezied from phenylalanine by three discrete enzymes, *viz*. PAL, C4H and PDC [**129**] (**Figure 2.7**).

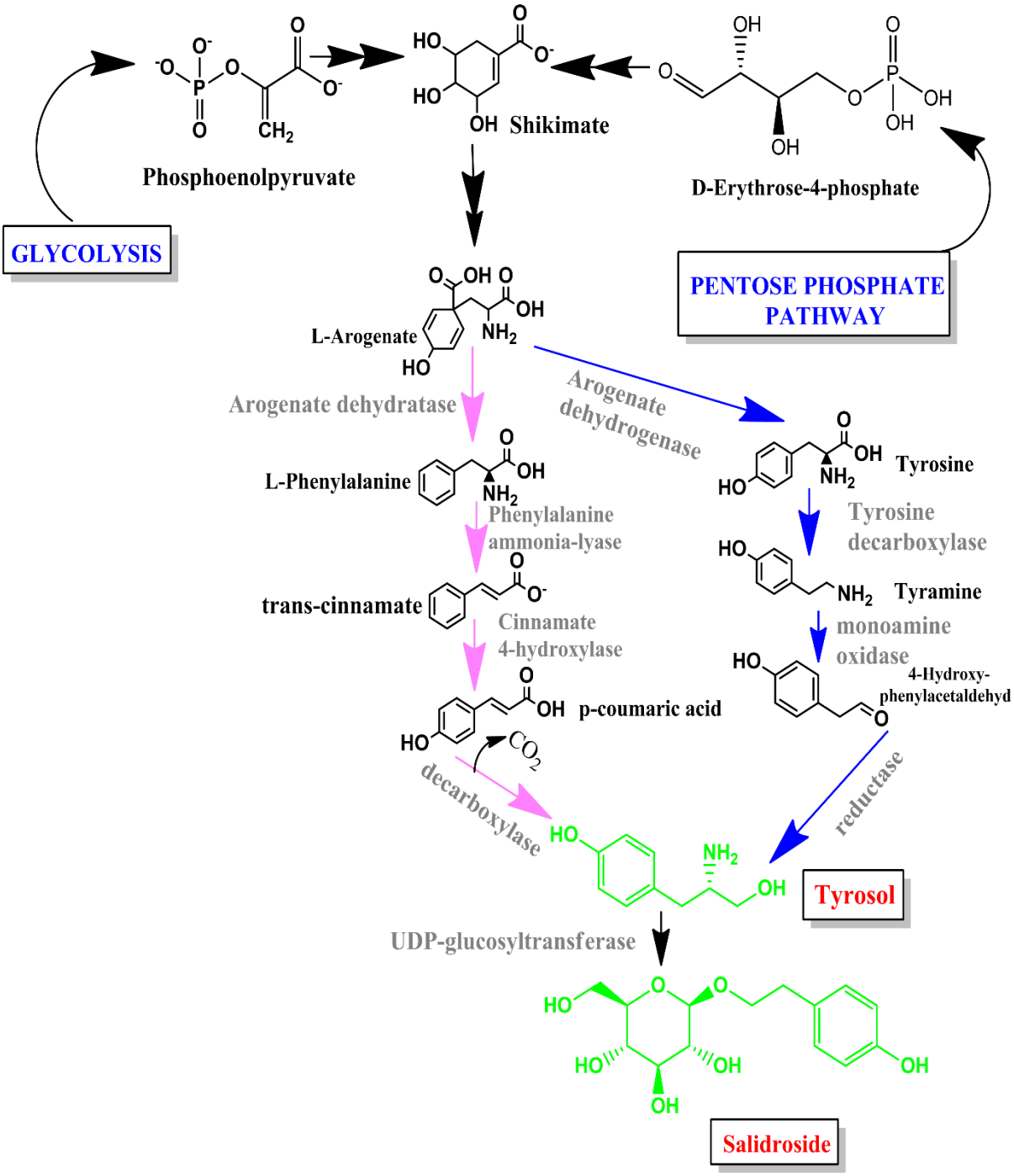


Figure 2.7: Biosynthetic pathway of Salidroside and Tyrosol. There are two different perspectives on the biosynthesis of salidroside. Pink arrow indicates that tyrosol is derived from phenylalanine **[128]**. Blue arrow indicates that tyrosol is derived from tyrosine **[129]**.

2.6 Identification of elite chemotypes

The global market of herbal based products is rapidly increasing due to their efficacy and safety in treatment of numerous human diseases **[130].** However, the quality and safety of

herbal based natural products have become a major concern for pharmaceutical industries, consumers and healthcare professionals [**131**]. Several researchers and phyto-pharmaceutical industries have reported significant variations in quality of plant-derived natural products, which could be primarily due to the diverse abiotic (environmental conditions, soil conditions, altitude, light and temperature) and biotic (plant age, developmental stage, tissue specificity and genetic constitution) factors [**132**]. The constant composition of major bioactive constituents is a crucial prerequisite for the authenticity and standardization of herbal based natural products. The phyto-pharmaceutical industries are constantly searching for elite plant chemotypes with higher contents of major bioactive constituents for improving the therapeutical potential of herbal products [**133**]. The screening and selection of elite chemotype is also highly beneficial for obtaining high producing cell lines [**134**]. *R. imbricata* is a traditional medicinal herb, which is utilized in several pharmaceutical and nutraceutical preparations. Salidroside and tyrosol are the major bioactive phenylethanoids of *Rhodiola* species, which are used as a biochemical marker for quality assurance and authenticity of *Rhodiola* based herbal formulations [**135**]. Several studies have reported substantial variations in content of major bioactive constituents in different species of *Rhodiola* collected from different geographical locations. Peschel et al. [**136**] also reported significant variations in content of phenylpropanoid in field-cultivated *Rhodiola rosea*. Zhao et al. [**137**] also reported a substantial variation in content of major active constituents in natural and *ex-situ* populations of *Rhodiola sachalinensis.* Lei et al. [**138**] found two major chemotypes of the essential oils in Yunnan (n-octanol- and geraniol-rich) and Tibetan (geraniol-rich) *Rhodiola crenulata* populations. Kosakowska et al. [**139**] reported a mixed tetrahydronootkatone/geraniol/trans-pinocarveol chemotype of *Rhodiola rosea.*

Rhodiola species also display a high level of morphological diversity in leaf margin dentation and plant dimensions throughout its distribution range (**Figure 2.8**) [**64].** Kurkin et al. [**122**] reported six morphotypes of *Rhodiola rosea* with varying yield and rosavin content. Several studies have also reported a high genetic diversity in different species of *Rhodiola* [**137**]. Hence, taking into consideration the high genetic and chemical variability in *Rhodiola* species, it is necessary to authenticate the *Rhodiola* species on molecular and biochemical level for ensuring the consistent efficacy and quality of *Rhodiola*-based herbal products.

Therefore, the literature review suggests that the screening of natural populations of *Rhodiola* from different geographical locations is necessary for the identification of

superior accessions with high content of major bioactive compounds, so as to increase its therapeutic efficacy and economical value. However, the screening and selection of the elite chemotypes of *R. imbricata* with higher contents of major bioactive phenylethanoids has not been reported to date.

Figure 2.8: *Rhodiola imbricata* growing at different geographical locations (**a**: Khardungla, **b**: Changla, **c:** Penzila) of trans-Himalayan Ladakh region.

2.7 Production of bioactive compounds from tissue culture of *Rhodiola*

Plants contains bioactive molecules of immense medicinal and commercial value [**140**]. These metabolites contribute to overall fitness of the plant in diverse ecological conditions [**141**]. They are used as dyes, insecticides, fragrances and pharmaceuticals [**141**].

The indiscriminate collection of medicinal plants from natural habitats for pharmaceutical and industrial endeavors has led to local extinctions, loss of genetic diversity and habitat destruction, and this in turn has resulted in the stringent restrictions in the harvesting of rare and endangered medicinal plant species form their natural habitats [**142**]. To overcome these constrains, plant cell cultures provides a potential platform for synthesis of bioactive metabolites of high industrial interest [**143**]. Shikonin by *Lithospermum erythrorhizon*, ginsenosides by *Panax ginseng* and paclitaxel by *Taxus* species are some of the important bioactive compounds that have been successfully produced through plant cell cultures [**144**]. Plant cell cultures offer several advantages over field cultivation and chemical synthesis, including sustainable, uniform and rapid supply of product, fast biomass proliferation, efficient downstream recovery of product, and production is continuous throughout the year without geographical, climatic, seasonal and environmental constraints [**30].**

The success of plant tissue culture depends upon the optimization of several physiochemical factors, including optimum concentrations of PGRs, carbon source and plant growth medium,

and appropriate temperature, light and humidity [**145**]. The plant cell cultures protocols need to be standardized for every plant species [**50**].

R. imbricata is a rich reservoir of bioactive compounds of immense medicinal and industrial value, but the natural population of *R. imbricata* is rapidly decreasing in its natural habitat. Plant cell culture offers potential alternative source of these commercially valuable bioactive compounds of *R. imbricata*. However, presently, a handful of reports are available related to the establishment of *in-vitro* cultures of *R. imbricata* for consistent production of bioactive compounds. Bhardwaj et al. [**147**] reported impact of various PGRs on direct regeneration from leaf explants of *R. imbricata*. The authors observed a shoot regeneration frequency (38.88 %) on MS+ 5 µM NAA+ 2.5 µM BA, whereas the maximum root induction was obtained with 0.5 µM NAA. The *in-vitro* propagated roots showed 1.49-fold higher amount of cinnamyl alcohol, as compared to the parent plant. However, the concentration of cinnamyl alcohol was remarkably low (0.001 %) in *in-vitro* propagated roots of *R. imbricata*. Therefore, suitable biotechnological approaches need to be followed to increase the production of bioactive metabolites of *R. imbricata*.

The plant tissue cultures studies on other species of *Rhodiola* have remained limited on the production of salidroside and rosavins (**Table 2.2)**. Furmanova et al. [**135**] reported the presence of salidroside and rosavins in callus cultures of *Rhodiola rosea*. Krajewska-Patan et al. [**147**] observed that cell cultures of *Rhodiola kirilowii* established on MS + 2 mg / l BA + 2 mg / l NAA + 1 mg / l AC showed maximum salidroside content (1.56 mg/100 g DW). The callus cultures of *Rhodiola sachalinensis* maintained on MS medium augmented with 5 mg/l BA + 2 mg/l IBA showed maximum salidroside content (3.27 + 0.18 mg/g DW) and the CCA suspension cultures obtained from these callus cultures showed highest production of salidroside (6.82 + 1.11 mg/g DW) in MS + 2 mg/l 2,4-D + 1 mg/l IBA + 3 mg/l BA [**148**]. Shi et al. [**149**] found that highest salidroside content (26.48 mg/g DW) was obtained in suspension cultures of *Rhodiola crenulata* established in MS + 0.1 - 0.5mg / lBA + 0.5 - 2 mg / l NAA .

Literature review revealed that there are no reports on the production of commercially important bioactive compounds, such as, flavones, terpenoids, carotenoids and phenolic acid in cell cultures of this medicinally important genus. Moreover, a limited progress has been

made so far to investigate the biosynthetic and bioactivity potential of cell culture system of *R. imbricata*. Since the biosynthetic potential of cell cultures of *R. imbricata* has never been investigated, the present study explored the feasibility of consistent and year-round synthesis of bioactive metabolites from cell culture system of *R. imbricata.*

Table 2.2: Bioactive compounds production in cell cultures of *Rhodiola* species

S. No	Plant species	Culture type	Explant	Media composition	Secondary metabolite	Reference
1	*R. crenulata*	Cell suspension culture		MS + 0.1-0.5 mg/l BA + 0.5-2 mg/l NAA	Salidroside (26.48 mg/g DW)	[149]
2	*R. kirilowii*	Callus culture	Cotyledons	MS + 2 mg/l BA + 2 mg/l NAA + 1 mg/l AC	Salidroside (1.56 mg/100 g DW)	[147]
3	*R. kirilowii*	Hairy root culture	Whole plantlets	LS + 1 ml/l Kin + 5 ml/l IBA + 10 mg/l AS	Rosavins (Not detected)	[158]
4	*R. rosea*	Compact callus suspension culture	Leaf	MS + 0.5 mg/l BA + 1 mg/l NAA	Rosavin (< 0.2 % of DW)	[155]
5	*R. rosea*	Callus culture	Leaf	MS + BA + NAA	Salidroside and Rosavins (present in traces)	[135]
6	*R. rosea*	Shoots and root culture	Seeds	NN + 0.01 mg/l NAA + 0.1 mg/l IAA	Salidroside and Rosavins (present in traces)	[135]
7	*R. rosea*	Shoots and root culture	Seeds	Shoots culture: MS + 2 mg/l zeatin or 2 mg/l BA	Salidroside (0.53 – 0.64 % of DW)	[159]

8	*R. sachalinensis*	Callus culture	Cotyledons	MS + 5 mg/l BA + 2 mg/l IBA	Salidroside (3.27 ± 0.18 mg/g DW)	[**148**]
9	*R. sachalinensis*	Callus culture	Leaf	$2B_5$ + 0.5 mg/l NAA+ 1 mg/l BA	Salidroside (0.41 % of DW)	[**151**]
10	*R. sachalinensis*	Callus culture		$2B_5$ + 1 mg/l NAA+ 5 mg/l BA	Salidroside (67.96 ± 3.41 mg/g DW)	[**152**]
11	*R. sachalinensis*	Compact callus suspension culture	Stem	MS + 0.3 mg/dm^3 NAA + 3 mg/dm^3 BAP	Salidroside (60 mg/ dm^3)	[**153**]
12	*R. sachalinensis*	Compact callus suspension culture	Stem	MS + 0.5 mg/l NAA + 1 mg/l BAP	Salidroside (0.68 % of DW)	[**154**]
13	*R. sachalinensis*	Compact callus suspension culture	Cotyledons	MS + 2 mg/l 2,4-D + 1 mg/l IBA + 3 mg/l BA	Salidroside (6.82 ± 1.11 mg/g DW)	[**148**]
14	*R. sachalinensis*	Cell suspension culture		$2B_5$ + 1 mg/l NAA+ 5 mg/l BA + 0.1 mg/l GA_3	Salidroside (90.3 ± 8.34 mg/l)	[**156**]

15	*R. sachalinensis*	Cell suspension culture	Leaf	$2B_5$ + 0.5 mg/l NAA+ 1 mg/l BA	Salidroside (0.12 – 0.41 % of DW)	[**151**]
16	*R. sachalinensis*	Cell suspension culture	Stem	MS + 0.3 mg/l NAA + 3 mg/l BAP	Salidroside (0.17 % of DW)	[**157**]
17	*R. sachalinensis*	Dispersed cell culture	Stem	MS + 0.5 mg/l NAA + 1 mg/l BAP	Salidroside (0.11 % of DW)	[**154**]
18	*R. quadrifida*	Callus culture		MS + 1 mg/l 2,4-D + 0.1 mg/l BA + 0.5 mg/l KT	Salidroside (0.28 % of DW)	[**150**]

MS: Murashige and Skoog medium, BA: Benzyl adenine, NAA: Naphthaleneacetic acid, AC: Adenine chloride, IBA: Indole-3-butyric acid, 2,4-D: 2,4-Dichlorophenoxyacetic acid, KT: Kinetin, GA3: Gibberellic acid, AS: Adenylyl sulfate, IAA: Indoleacetic acid, DW: Dry weight.

2.8 Elicitation

Elicitation is a useful approach to induce the production of bioactive molecules in plant cell culture using elicitors [**160**]. The elicitors are biofactors or chemicals from abiotic and biotic sources that triggers a series of morphological, molecular, biochemical and physiological changes in plants [**161]**. The abiotic elicitors include physical agents and chemical factors, such as, heat shock, hydrostatic pressure, hyperosmotic stress, jasmonic acid, salicylic acid, silver nitrate, cobalt nitrate, vanadate and sodium acetate etc. [**162**]. The biotic elicitors include plant-based elicitors, fungal-, animal-, bacterial- and algae-derived elicitors, such as, chitosan, pectin, yeast extract, alginate and fungal-derived cerebosides etc. [**162**]. Several studies have shown that the abiotic and biotic elicitors trigger the secondary product formation via stimulating metabolic networks [**46**]. *Alternaria alternata* activates the G-protein and protein kinases in *Citrus limon* that downstream regulates the expression of PAL genes, resulting in accrual of the umbelliferone and scoparone [**163**]. The addition of jasmonic acid, vanadyl sulphate, arachidonic acid, lanthanum salts, salicylic acid and fungal

extracts, alone or in combinations, results in increased *in-vitro* synthesis of taxane in *Taxus* species [**164**]. The cell culture of *Rhodiola sachalinensis* elicited with 50 mg/l of *Penicillium* sp. extract increased the production of salidroside (7.8 mg/g DW) [**165**]. The salicylic acid (5 – 45 mg/l) treatment increased the salidroside content (8.9 mg/g DW) in compact callus suspension culture of *Rhodiola sachalinensis* [**148**]. Elicitors are used an important biotechnological tool to study the biosynthesis of known and novel secondary metabolites [**162**]. The supplementation of *Aspergillus niger* extract (0.05 g/l) in the medium improved the accrual of salidroside (6.2 mg/g DW) in hairy root culture of *Rhodiola sachalinensis* [**166].** The *Rhizopus delemar* extract treatment increased the production of salidroside (2.6 mg/g DW) in cell culture of *Rhodiola sachalinensis* [**165**]. The addition on spermidine improved the salidroside yield (69 + 2.88 mg/l) in cell culture of *Rhodiola sachalinensis* [**156**]. The success of elicitation relies on several factors, including type and specificity of elicitors, exposure time and time of addition of elicitors, media composition, secondary metabolite of interest and cell line **[160].**

The elicitor induces an array of defense responses that lead to the accretion of defensive metabolites in cell cultures [**46**]. Elicitor perception results in dephosphorylation and phosphorylation of proteins, rise in intercellular calcium, influx of protons and efflux of potassium and chlorine, intracellular acidification, plasma membrane depolymerization, ROS production and activation of G-proteins, CDPKs, PPs, PKC, NOX, APRX and MAPKs cascade, that eventually lead to the activation of transcription factors, which regulates the expression of defense-related genes involved in the biosynthesis of secondary metabolites [**46**].

2.8.1 Light quality

Light has long been used for improving growth, development and bioactive product formation in *in-vitro* cultures [**47**]. Light quality is a vital abiotic factor stimulating the growth and bioactive metabolites formation in *in-vitro* cultures [**47**]. Several authors have extensively reported the molecular and physiological basis of photoinduced processes [**167**]. LEDs have evolved as an economically viable source for *in-vitro* cultures [**50**]. LEDs are semiconductors based on the principle of electroluminescence [**50**]. Several researchers have demonstrated the stimulatory impact of LEDs on plant growth and secondary metabolism [**54**]. The several advantages of LEDs in plant *in-vitro* cultures have been extensively

reviewed by numerous researchers [**168**]. The various physiological and metabolic processes in plants are regulated by distinct light spectrum sensed through diverse photoreceptors [**48**]. Plants have evolved transcriptional networks that regulate developmental processes in response to light [**169**]. Light induces an immense reprogramming of the plant transcriptome [**169**]. Several researchers have reported that light regulates and alters the transcription, translation and stability of mRNA [**170**]. Light is also a major energy source for plant photosynthesis [**169**]. Several studies suggested that the light quality significantly regulates the expression of photosynthesis-related genes [**171**].

Light quality has a strong influence on plant morphogenesis, photosynthesis, and other physiological processes [**172**]. The spectral irradiance of sunlight is a mixture of photosynthetically active radiation (400-700 nm), far-red (690-760 nm) and UV radiation (100-400 nm) [**173**]. The light quality regulates the plant growth and physiological processes through selective activation of photoreceptors, such as, cryptochromes and phototropins by blue light and phytochromes by red and far-red light [**174**]. Light quality affects the synthesis of amino acid, sugars, phytohormones and nucleic acids by regulating the gene expression of various metabolic pathways [**174**]. Several authors have reported a positive correlation between the light quality and expression of *CAB* genes [**175**]. Light spectra regulates the growth in several plant cell cultures, such as, biomass accreation is significantly altered by light quality in cell cultures of *Prunella vulgaris* [**54**], *Artemisia absinthium* [**55**], *Stevia rebaudiana* [**176**] and *Thevetia peruviana* [**177**]. Light quality alters the plant physiological processes, including photosynthesis and photomorphogenesis by the induction of signal response elements and biochemical variations **[178].** Several plant genes involved in photosynthesis have been found to alter their expression patterns in response to light [**179**]. Wang et al. [**173**] found that the light quality has significant effect on the expression and activity of the calvin cycle enzymes. Light quality also affects the phytohormones metabolism, which in turn regulates the plant growth and development [**180**].

Light spectra has a stimulatory influence on synthesis of bioactive molecules in plant cell cultures [**35**]. Light spectra have been implicated in controlling the biosynthetic pathways of bioactive compounds, including phenolic compounds (flavonoids, anthocyanins and phenolic acid), alkaloids and terpenoids [**180**]. This regulation is achieved by specific activation of diverse class of photoreceptors, transcription factors, enzymes expression and gene modulation [**181**]. It is also significantly regulated by degradation and compartmentalization

of signaling intermediates involved in regulating the expression of genes associated with secondary metabolitsm [**182**]. Different light spectra have been utilized for enhancing the biosynthesis of bioactive molecules in plant cell cultures, such as, production of phenolic acid in *Haplopappus* and *Populus* species [**183**], isoflavones in *Cyclopia subternata* [**184**], flavonoids in *Alternanthera* [**185**] and terpenoids in *Achillea millefolium* [**186**]. Liu et al. [**179**] found that the expression of *PAL1, 4CL1, LDI2, HMGR,* and *CYP98A6* was regulated by different lights at transcriptional level in *Onosma paniculatum* cell cultures by phytochromes and blue light receptors systems. Light can differentially regulate the plant secondary metabolism through activation of distinct light-responsive metabolic pathways [**35**]. Though different lighting conditions have been extensively utilized for enhancing the biosynthesis of bioactive molecules in several plant cell cultures, yet these have not been utilized, till date, for enhancing the biosynthesis of bioactive molecules in cell cultures of *R. imbricata.*

2.8.2 Jasmonic acid

Jasmonic acid methyl ester was discovered in 1962 in *Jasminum grandiflorum* [**187**]. which led to an era of subsequent studies that unraveled the versatile functions of jasmonates. Jasmonates are a family of cyclopentanone compounds that regulate many developmental and physiological processes in plants, including fruit ripening, floral and pollen development, root growth, senescence, tuberization, tendril coiling and fungi arbuscular mycorrhizal association [**57, 188**]. Jasmonates are also involved in the activation of plant defense responses during biotic and abiotic stresses and it has long been observed to elicit the secondary metabolite pathways in plants [**57**]. The JA is biosynthesized through the action of stimuli-specific lipases [**189**]. The production of JA is controlled by positive feedback, tissue specificity and substrate availability [**189**]. MAPK cascades and calcium are also associated in controlling the biosynthesis of JA [**189**]. JA act as a signal transducer that perceives and transduces the external signal, which results in the synthesis of bioactive metabolites [**190**]. The breakthrough in JA signaling was the discovery of negative regulation of JA-mediated gene expression by JAZ proteins [**189**]. JA-induced signal transduction primarily involves synergistic action of JA-Ile and COI1 complex on degradation of JAZ proteins through 26S-proteasome, thereby relieving the negative repression of JAZ proteins on transcription factors, including, MYCs, AP2 and several bHLHs, that are in turn involved in up-regulating the expression of secondary metabolites biosynthetic genes. The wound response in *Solanum*

lycopersicum is regarded as best signal-transduction pathways of jasmonic acid, in which JA negatively affects the herbivore performance through up-regulating the expression of genes encoding proteinase inhibitors. [**200**]. JA also shows synergistic and antagonistic interactions with PGRs, such as gibberellins, abscisic acid, and salicylates.

Gundlach et al. [**58**] for the first time reported an intercellular increase in levels of JA upon elicitation in cell cultures of *Eschscholtzia californica* and *Rauvolfia canescens*. Several studies have reported that exogenous application of JA could induce endogenous JA biosynthesis and subsequently, endogenous JA triggers the plant secondary metabolism [**46**]. This has resulted in great rise in the applications of JA for *in-vitro* production of bioactive compounds [**192]**. JA has been reported to induce synthesis of plumbagin in hairy roots of *Plumbago indica* [**61],** anthocyanin in *in-vitro* cultures of *Vitis vinefera* [**193**], phenylpropanoids and naphtodiantrones in cell suspensions of *Hypericum perforatum* [**60**] and coumarin production in *Mikania laevigata* [**194**]. Thus, it can be inferred that modulation of secondary metabolites production through JA treatment is a lucrative option, but, information regarding the influence of jasmonic acid treatment on accrual of bioactive molecules in cell cultures of *R. imbricata* is not available as of today.

2.9 Bioactivities in plant cell cultures

2.9.1 Antioxidant activity

Antioxidants are emerging as prophylactic and therapeutic agent [**195**]. Plants contains natural antioxidants, which act as reducing agents, hydrogen donators, oxidant and free radical scavengers [**196].** Natural antioxidants exert several beneficial effects on human health, including protection against the ROS-mediated inflammatory diseases (lupus erythematous and glomerulonephritis), neurological disorders and other degenerative diseases [**197**]. Plants primarily produce antioxidants compounds to keep reactive oxygen species and other free radicals at a basal non-toxic level [1**98]**. The low molecular weight non-enzymatic antioxidant compounds, including ascorbic acid, glutathione, carotenoids, tocopherol and phenolic compounds, generally provides the protection against reactive oxygen species (ROS), reactive nitrogen species (RNS) and other free radicals [**32**]. However, synthesis of these antioxidants is highly fluctuating and depends on several environmental, and genetic factors [**132**]. Moreover, there is a limited availability of plant material from natural source due to overexploitation, climatic and other abiotic and biotic factors [**142**]. Therefore, alternative approaches have been used for biosynthesis of natural antioxidants. Plant cell

culture are the most effective biotechnological platform for substantial synthesis of antioxidants [**32**]. The phenolic compounds and isoprenoids are the major chemical classes of antioxidant compounds obtained from plant cell cultures [**32**]. The phenolic compounds, such as, anthocyanidins, flavanons, isoflavonoids, flavanols, flavones and proanthocyanidins derived from plant cell cultures have pronounced antioxidant activities [**32**]. The antioxidant properties of phenolic compounds usually depend on the hydroxylation pattern and structural diversity [**32**].

The synthesis of antioxidant compounds via plant cell cultures are often constrained by low and inconsistent yields. Recently, several approaches have been proposed for increasing the biosynthesis of antioxidants in cultured plant cells. Elicitation is extensively utilized for enhancing the accrual of antioxidant compounds in *in-vitro* cultures [**32**]. Biotic elicitors, such as, yeast extract increased the production of antioxidant rosmarinic acid and phenolics in hairy roots of *Salvia miltiorrhiza* [**199**]. Chitin treatment increased the synthesis of several novel flavonoids in cell cultures of *Cephalocereus senilis* [**200**]. Abiotic elicitors, including Lanthanum promoted the biosyntheis of antioxidant silymarin in cell cultures of *Silybum marianum* by interfering with cellular calcium signalling [**201**]. The TPC, TFC and antioxidant potential increased substantially in *in-vitro* cultures of *Fagonia indica* [**202**] and *Cyclopia subternata* [**184**] after treatment with different light spectra. UV-C treatment increased the synthesis of phenolics and antioxidant capacity in *in-vitro* cultures of *Linum usitatissimum* [**203**]. Jasmonic acid treatment enhanced the accrual of antioxidants in *in-vitro* cultures of *Artemisia absinthium* [**204**]. Jasmonic acid also increased the synthesis of TPC, tfc and antioxidant capacity in callus of *Opuntia* species [**205**]. Salicylic acid treatment enhanced the production of antioxidant norswertianin in *in-vitro* cultures of *Gentiana dinarica* [**206**]. Bioreactor technology has been utilized for the scale-up synthesis of several antioxidants including production of rosmarinic acid in cell cultures of *Anchusa officinalis* **[207]** and anthocyanins in cell suspension cultures of *Aralia cordata* [**208**]. The rapidly increasing metabolic engineering approaches offers a potential platform for enhancing the synthesis of antioxidants in plant cell cultures [**32**]. The antioxidant potential can easily be evaluated using several *in-vitro* based assays, including DPPH, TEAC, FRAP and nitric oxide scavenging method [**209**]. The *in-vitro* assay, such as, free radical scavenging assay is based on the type of chemical reactions involved *i.e.* HAT reaction-based methods and SET reaction-based methods **[210].** Therefore, keeping in view, the enormous health-

promoting benefits of natural antioxidants, the discerning of antioxidant activities in cell culture system of *R. imbricata* would be of great benefit.

2.9.2 Antimicrobial activity

Plant contains a diverse array of secondary metabolites, which have been considered to have antimicrobial properties [**211**]. Plant produces phytoalexins, antimicrobial proteins, lytic enzymes and antimicrobial compounds to protect themselves against microbial invasions [**212**]. The antimicrobial activity is instrumental in numerous applications, including natural therapies and pharmaceuticals [**213**]. The phenolic compounds, lectins and terpenoids are major class of antimicrobials in plants [**211**]. The toxicity of phenolic compounds against microorganisms are generally ascribed to the number and site(s) of hydroxyl groups on phenolic compounds [**214**]. These phenolic compounds inhibit the microbial enzymes through non-specific interactions with the proteins [**215**]**.** The phenolic compounds may also disrupt microbial membranes [**216**]. The terpenoids compounds forms a complex with microbial membranes and disrupts it, thereby inhibiting the growth of microorganism [**211**]. The diffusion methods, such as, agar well diffusion and disk-diffusion, and TLC, bio-autoradiography, and the dilution methods, including broth and agar dilution method are widely used methods for screening *in-vitro* antimicrobial activities [**217**].

Recently, there has been tremendous increase in the search and use of plant-derived natural drugs for treatment of infectious diseases caused by pathogenic microorganisms. This upsurge in plant-derived drugs could be due to the side effects, ineffectiveness and high production cost of traditional antibiotics [**211**]. However, due to this upsurge in natural plant-based antimicrobials, the population of high value medicinal plant species are rapidly decreasing in the wild [**218**]. Plant cell cultures have emerged as a promising alternative source of antimicrobial compounds, namely in cultures of *Crataegus azarolus* [**219**], *Salvia corrugata* [**220**], *Lavendula angustifolia* [**212**], *Knautia sarajevensis* [**221**] and *Alkanna orientalis* [**222**]. Thus, keeping in view, the increasing incidence of microbial resistance and efficacy of plant-derived natural products against pathogenic microorganisms, the identification of antimicrobial activities in cell culture of *R. imbricata* is highly desirable.

Overall, the review of literature of *R. imbricata* has revealed the following research gaps:

- The selection of elite chemotypes is highly beneficial for obtaining high producing cell lines. However, there is no reports on the identification of elite chemotypes of *R. imbricata*.
- Plant cell culture system offers promising platform for synthesis of bioactive molecules. However, till date, no study has been done to investigate the biosynthetic potential of *R. imbricata* cell culture system for synthesis of bioactive compounds.
- The stimulatory effect of light quality and jasmonic acid on the secondary metabolites production and bioactivities has been well-documented in several plant cell cultures, but, so far, such elicitation strategies have never been applied to cell cultures of *R. imbricata.*

CHAPTER 3

MATERIAL AND METHODS

3.1 Plant material

A total of 18 plant accessions (6 accessions from each high-altitude pass) of *R. imbricata* were collected from Penzila, Changla, and Khardungla pass of Ladakh region (**Figure 3.1**) and established in the MAP garden of DIHAR, Leh, India (11482 ft, 34.170ºN & 77.566ºE). The plants were identified by the BSI, Dehradun (Accession no. – 117062). The details of *R. imbricata* accessions are provided in **Table 3.1**.

Table 3.1: Details of *Rhodiola imbricata* accessions collected from different geographical locations of Ladakh region.

S.No	ID	Location	Latitude (N)	Longitude (E)	Altitude (m)
1	CA1	Changla	34.255	78.130	5355 ± 6.6
2	CA2	Changla	34.273	78.136	5350 ± 6.6
3	CA3	Changla	34.255	78.137	5352 ± 5.7
4	CA4	Changla	34.255	78.138	5336 ± 7.2
5	CA5	Changla	34.255	78.14	5340 ± 4.6
6	CA6	Changla	34.257	78.139	5339 ± 4.6
7	KA1	Khardungla	34.289	77.875	5663 ± 9.2
8	KA2	Khardungla	34.277	77.876	5614 ± 2.8
9	KA3	Khardungla	34.292	77.878	5618 ± 3.0
10	KA4	Khardungla	34.282	77.597	5632 ± 6.2
11	KA5	Khardungla	34.296	77.88	5638 ± 5.7
12	KA6	Khardungla	34.294	77.883	5646 ± 6.1
13	PA1	Penzila	33.856	76.562	4121 ± 13.3
14	PA2	Penzila	33.894	76.588	4213 ± 5.9
15	PA3	Penzila	33.881	76.575	4354 ± 11.4
16	PA4	Penzila	33.959	76.463	4321 ± 9.3
17	PA5	Penzila	33.959	76.454	4121 ± 8.9
18	PA6	Penzila	33.957	76.462	4123 ± 10.9

Figure 3.1: Collection of *Rhodiola imbricata* from different geographical locations (Khardungla, Changla and Penzila pass) of trans-Himalayan region of Ladakh.

3.2 RP-HPLC analysis

3.2.1 Sample preparation and extraction procedure

All the samples were oven dried (40 °C), pulverized and suspended in 100% methanol (1:15 w/v; HPLC grade, Sigma). Then, ultrasonicated (3 x 30 min at 25 ℃) and centrifuged (5000 g at 4 ℃ for 5 min). The supernatant was collected and filtered (0.22 µm filter). The samples were then diluted 15-fold with 100% methanol and used for the quantification of phenylethanoids by RP-HPLC.

3.2.2 RP-HPLC method

The phenylethanoids were quantified using Agilent 1260 HPLC system fitted with DAD detector. The best chromatographic conditions used were as follows: Agilent eclipse C18 column (4.6 × 100 mm, 3.5 µm); column temperature (25 ℃); sample injection volume (5 µl); λ_{max} (225 nm); mobile phase (solvent A (H_2O) and solvent B (CH_3CN (HPLC grade,

Sigma)); flow rate (1 ml / min); 0-6 min (85 % A and 15 % B; isocratic), 7-14 min post run wash (4 min (100 % B) and 4 min equilibration (85 % A and 15 % B). The chromatogram was interpreted using Agilent EZ chrom software (A.04.04). The linear regression equations obtained from four-point calibration of standards was used for quantification of salidroside and tyrosol (mg/g DW) in processed samples of *R. imbricata*.

The following formula was used for assessment of phenylethanoids yield (mg/l):

$$Phenylethanoids\ yield\ (mg\ /\ l) = Dry\ weight\ (g\ /\ l) \times Phenylethanoids\ content\ (mg\ /\ g)$$

3.2.3 RP-HPLC method validation

3.2.3.1 Calibration curves

A 1 mg/ml stock solution of salidroside and tyrosol (≥ 95 % (LC/MS), Sigma) was prepared in 100 % methanol and five concentration levels were diluted from the stock. Concentrations of standard solutions were 1, 5, 25, 125 and 625 µg/ml.

3.2.3.2 LOD and LOQ

The LOD and LOQ were recorded as per the guidelines of ICH at a Signal/Noise of about 3 and 10, respectively [**223**].

3.2.3.3 Precision and Accuracy

Inter and Intra-day precision were performed for evaluating method precision. Intra-day precision of the method was computed by three replicate measurements, each of five different concentrations preparations of standards in single day, whereas, for inter-day test, the same intra-day procedure was followed for consecutive two days and the results were expressed in RSD %. The accuracy of the method was computed on the basis of method of standard addition and recovery for which three replicates of five different concentration of standards was added in the blank matrix and the results was expressed in terms of RSD %.

3.3 Establishment of callus cultures

The stem and leaf explants of *R. imbricata* were washed under water (15 min), treated with STRS (0.02 % w/v; 10 min), Bavistin (0.02 % w/v; 10 min) and washed with autoclaved water. Then, surface sterilized with EtOH (70 %; 2 min), NaClO (10 % v/v, 10-15 min), washed with water and dried on blotting paper.

Then, the disinfected explants were inoculated on MS media [**224**] fortified with 30 g/l sucrose, 8 g/l agar and various concentrations of BA (0-5 mg/l) and NAA (0-5 mg/l) (pH: 5.75). The cultures were incubated under white light at 22.45 µmol $m^{-1}s^{-2}$ intensity, 25 ± 1 °C, 65% RH and 16-h photoperiod. The CIF was assessed daily and calculated as follows:

$$Callus\ induction\ frequency\ (\%) = \frac{Total\ number\ of\ explants\ induced\ callus}{Total\ number\ of\ explants\ inoculated} \times 100$$

3.3.1 Effect of PGRs and explant type on biomass accumulation

The influence of various concentrations of BA (0-3 mg/l) and NAA (0-3 mg/l) and explant type (leaf and stem (in triplicates)) on FW and DW accumulation in callus cultures established under optimized conditions (**Section 3.3**) were recorded after 30 days.

3.3.2 Effect of PGRs on production of phenylethanoids

The influence of various concentrations of BA (0-3 mg/l) and NAA (0-3 mg/l) on phenylethanoids accumulation in callus cultures (in triplicates) established under optimized conditions (**Section 3.3**) were recorded after 30 days.

3.3.3 Growth and production kinetics

The periodic evaluation of growth (FW and DW) and phenylethanoids production in callus cultures (in triplicates) established under optimized conditions (**Section 3.3**) were performed at 0, 5, 15, 21, 25 and 30 days.

3.3.4 Analytical methods

3.3.4.1 Growth determination

The callus cultures were harvested, pressed on blotting paper and weighed for assessing FW (g/l). Then, the callus was oven dried (40 °C) and weighed for assessing DW (g/l). During

exponential phase, the SGR (μ (days^{-1}) and DT (td (hours)) were calculated using Panda et al. [**225**] method. The GI were calculated using Ketchum et al. [**226**] method as follows:

$$GI = \frac{W_tF - W_tI}{WtI}$$

Where GI = Growth index, *Wt F* = Final DW, *Wt I* = Initial DW.

3.3.4.2 Biochemical analysis

3.3.4.2.1 Sample preparation and extraction procedure

All the samples were prepared and extracted using previously established method (**Section 3.2.1)**.

3.3.4.2.2 Quantification of phenylethanoids by RP-HPLC

The phenylethanoids were quantified using previously established HPLC method (**Section 3.2.2)**.

3.3.4.2.3 Determination of total phenolic content and ascorbic acid content

The AAC and TPC were calculated using Sánchez-Rangel et al. [**227**] method. The absorbance was measured at 765 nm using microplate reader (SpectraMax M5) and SoftMax Pro 6.1 software. A five-point calibration curve was plotted using ascorbic acid (0.02- 0.1 mg/ml; (AA)) and chlorogenic acid (0.02- 0.125 mg/ml; CHA) as standard and TPC and AAC was calculated from the regression equation:

(TPC: y = 0.0012x + 0.0103, r^2 = 0.990 (CHA) and AAC: y = 0.0006x – 0.0064, r^2 = 0.998 (AA)) and specified as mg CHA/g DW and mg/g DW, respectively.

The following formula was used for assessment of AAP (mg/l) and TPP (mg/l):

$Ascorbic\ acid\ production\ (mg/l) = Dry\ weight\ (g/l) \times AAC\ (mg/g)$

$Total\ phenolic\ production\ (mg/l) = Dry\ weight\ (g/l) \times TPC\ (mg\ CHA/g)$

3.3.4.2.4 Determination of total flavonoid content

The TFC was calculated using Zhishen et al. [**228**] method, with minor modifications. Extract (1ml (1mg/ml)) was added to H_2O (4 ml) and $NaNO_2$ (0.3ml (5 % w/v)). Then, $AlCl_3$ (0.3ml (10 % w/v)) was added 5 min later. After 6 min, NaOH (2ml (1 M)) and H_2O (3.4 ml) was added. The absorbance was measured at 510 nm and a five-point calibration curve was plotted using rutin (0.025- 0.125 mg/ml) as standard. TFC was calculated from the regression equation ($y = 0.0009x + 0.0011$, $r^2 = 0.998$) and specified as mg RE/g DW.

The following formula was used for assessment of TFP:

$$Total\ flavonoid\ production\ (mg/l) = Dry\ weight\ (g/l)\ \times TFC\ (mg\ RE/g)$$

3.3.4.2.5 Determination of total flavonol content

The total flavonol content was calculated using Kumaran and Karunakaran method [**229**]. Extract (2ml (1mg/ml)) was added to $AlCl_3$ (2 ml in 2 % ethanol) and $NaNO_3$ (3ml (50 g/l)). The absorbance was measured at 440 nm after incubating the mixture at 25 °C for 150 min. A five-point calibration curve was plotted using quercetin (0.025- 0.125 mg/ml) as standard and total flavonol content was calculated from regression equation ($y = 0.0015x - 0.019$, $r^2 = 0.995$) and specified as mg QE/g DW.

3.3.4.2.6 Determination of carotenoids

The carotenoids content was calculated using Lichtenthaler and Buschmann method [**230**]. The absorbance was measured at 470 nm, 652.4 mm and 665.2 nm. The following formula was used for assessment of carotenoids and specified as mg/g FW.

$$Cartenoids\ (mg/g\ FW) = \frac{1000\ A_{470} - 1.63\ (16.72\ A_{665.2} - 9.16\ A_{652.4}) - 104.96\ (34.09\ A_{652.4} - 15.28\ A_{665.2})}{221}$$

3.3.4.3 GC-MS analysis

3.3.4.3.1 Extraction procedure and sample preparation for GC-MS analysis

After thirteen successive subcultures, the callus was harvested and washed with double distilled water. Subsequently, it was shade dried at room temperature for 10 days. Then the dried callus was ground to fine powder by mortar and pestle. The callus powder (15 g) was then sequentially extracted in four different solvents i.e. n-hexane, chloroform, ethyl acetate and methanol. The samples were extracted in a Soxhlet apparatus at 40°C. Finally, the extracted fractions were concentrated using rotavapor (Büchi R-205, Switzerland). The 20 mg of extracted fractions were dissolved in the respective solvents, vortexed and filtered (0.22 µm PTFE syringe filter) and stored at -20°C.

3.3.4.3.2 GC-MS method

GC–MS analysis was conducted on Shimadzu GC-2010 Plus GC-system coupled with GC-MS-QP 2010 Ultra MS. The following GC-MS conditions were followed: (RTx-5Sil MS column (30 m x 0.25 mm x 0.25 µm), sample (1 µl, split mode, splitting ratio (1:5), inlet temperature (280°C), oven temperature (50°C, 2 min , then raised to 300°C at 10°C per minute and maintained for 13 min , Helium (He) carrier gas (flow rate: 1 ml/min), ion source temperature (200°C) and interface temperature (300°C)). Mass spectra was acquired with EI mode at 70 eV and 40-1000 m/z range. The mass spectra were acquired at an interval of 0.3 s . GC-MS solution software was used to process the chromatographic and spectrometric data. The compounds were identified based on the comparison of mass spectra with mass spectral database of NIST/EPA/NIH (NIST 11) and Wiley 8.0 (Wiley, USA). The phytochemical database was used for analyzing the bioactivity of phytochemicals assessed through GC-MS [**231**].

3.4 Establishment of CCA Suspension cultures

CCA suspension culture were initiated using CCA (20 g FW) in 250 ml MS media containing 30 g/l sucrose + 3 mg/l NAA + 3 mg/l BA (pH: 5.75; in triplicates). The cultures were maintained on orbital shaker (110 rpm) at 25 ± 1 °C in continuous light and regularly sub-cultured at 10 days interval.

3.4.1 Effect of culture media components on biomass accumulation and production of phenylethanoids in CCA suspension cultures

The following chemical factors were varied under defined culture conditions (**Section 3.3**) to examine their influence on FW, DW and accumulation of major phenylethanoids in CCA ((1 g/20 ml FW) suspension culture over 10 days (in triplicates):

(i) Basal media (MS, 1/2 MS, B5 [**232**] and 1/2 B5), (ii) Carbon source (maltose, sucrose, fructose and glucose (30 g/l)), (iii) Sucrose concentrations (10, 30, 50, 70 g/l) and (iv) PGRs (BA (0-3 mg/l) and NAA (0-3 mg/l); periodic evaluation at 2 days interval).

3.4.2 Analytical methods

3.4.2.1 Growth determination

The CCA suspension cultures were harvested, washed and filtered (vacuum driven filtration) and weighed for assessing FW (g/l). Then, the callus was over dried (40 ℃) and weighed for assessing DW (g/l).

3.4.2.2 Biochemical analysis

3.4.2.2.1 Sample preparation and extraction procedure

All the samples were prepared and extracted using previously established method (**Section 3.2.1)**.

3.4.2.2.2 Quantification of phenylethanoids by RP-HPLC

The phenylethanoids were quantified using previously established HPLC method (**Section 3.2.2)**.

3.5 Elicitation experiments

3.5.1 Establishment of callus cultures under different light conditions

The callus was established under optimized culture conditions (**Section 3.4**) and following LEDs were used: red (610-715 nm, 100%), green (480-670 nm, 100%), blue (380-560 nm, 100%) and RGB (Red: Green: Blue; 40 %: 40 %: 20 %) at 33 ± 8.25 µmol $m^{-1}s^{-2}$ intensity and 25 ± 1℃, inside a Fytoscope (in triplicates). The

Fytoscope was equipped with a photoperiodic and intensity controller (FS 130, Czech Republic). The cultures were also incubated in white light as control under optimized culture conditions (**Section 3.4**). The periodic evaluation of growth (FW and DW) and phenylethanoids production were performed at 0, 5, 15, 21, 25 and 30 days. The AAC, TFC and TPC, and bioactivities (antioxidant and antimicrobial activity) were determined after 30 days. The growth was calculated using previously established method (**Section 3.3.4.1)**. All the samples were prepared and extracted using previously established method (**Section 3.2.1)**. The phenylethanoids were quantified using previously established HPLC method (**Section 3.2.2)**. The AAC, TFC and TPC were calculated using previously established method (**Section 3.3.4.2.3** and **3.3.4.2.4)**.

3.5.2 Effect of JA Treatment

The time-course influence of JA (0, 5 and 100 µM; 95 % v/v methanol (0.22 µM filter sterilized)) elicitation on FW and DW accumulation, and phenyethanoids, TFC, AAC and TPC production, and DRSA and TAC in CCA suspension cultures (1 g/20 ml) maintained in optimized culture conditions (**Section 3.4**) were calculated at 2 days interval for the duration of 6 days (in triplicates). The growth was calculated using previously established method (**Section 3.3.4.1)**. All the samples were prepared and extracted using previously established method (**Section 3.2.1)**. The phenylethanoids were quantified using previously established HPLC method (**Section 3.2.2)**. The AAC, TFC and TPC were calculated using previously established method (**Section 3.4.2.3** and **3.4.2.4)**.

3.6 Determination of bioactivities

3.6.1 Sample preparation and extraction procedure

All the samples were prepared and extracted using previously established method (**Section 3.2.1)**.

3.6.2 Antioxidant activity

3.6.2.1 DPPH free radical scavenging activity

DRSA was calculated using Yesmin et al. [**233**] method, with slight modifications. Extract (200 µl (0.125 mg/ml)) was added to 3 ml of DPPH solution (0.004 % w/v) and thoroughly

mixed. Absorbance was measured at 517 nm after incubating the mixture at 25℃ for 30 min in dark. Quercetin and BHA was used as standard.

The following formula was used for assessment of DRSA:

$$DRSA\ (\%)\ =\ \left[\frac{Abs\ Control - Abs\ Sample}{Abs\ Sample}\right] \times 100$$

Where,

$Abs_{Control}$ = DPPH absorbance.

Abs_{Sample} = DPPH absorbance with extract.

3.6.2.2 Total antioxidant capacity

TAC was calculated using Prieto et al. [**234**] method. Absorbance was measured at 695 nm after incubating the mixture at 25℃. A five-point calibration curve was plotted using quercetin (0.025- 0.125 mg/ml) as standard and TAC was calculated from the regression equation ($y = 0.0019x - 0.025$, $r^2 = 0.995$) and specified as mg QE/g DW. Ascorbic acid and BHA was used as standard.

3.6.3 Antimicrobial activity

The antimicrobial activities were examined against *Escherichia coli* ATCC 25922 and methicillin-resistant *Staphylococcus aureus* ATCC 43300 using agar well diffusion method, and by determining MIC and MBC.

3.6.3.1 Well diffusion method

The antimicrobial activities were assessed using agar well diffusion method [**235**]. A 10^6-10^8 cells/ml test samples (100 µl) was spread on nutrient agar plates and 50 µl of extract (40 mg/ml) was suspended in agar wells (5 mm) created by cork borer. Then, the zone of growth-inhibition (mm) was recorded in plates after 18-24 h incubation at 37℃. Methanol and gentamicin were utilized as controls.

3.6.3.2 Minimum inhibitory concentration

CLSI guidelines were followed for assessment of MIC [**236, 237**]. A two-fold diluted extracts (0.15-40 mg/ml (50 µl)) was added to 50 µl of test samples and suspended in microtiter plates. Then, the turbidity was examined after incubating the plates for 18-24 h at 37°C.

3.6.3.3 Minimum bactericidal concentration

CLSI guidelines were followed for assessment of MBC [**236, 237**]. Test samples (100 µl) from microtiter plates were spread on agar plates. Then, the cell viability was recorded in plates after overnight incubation at 37°C.

3.7 Statistical analysis

All the experiments were performed in a CRD research design, analysis was executed in triplicates and results were expressed as mean ± SD. The differences in between and among group means were calculated using one-way ANOVA and Bonferroni test, respectively at $p \leq 0.05$. The significant level for Pearson correlation was set at $p \leq 0.05$ and $p \leq 0.01$, SPSS (USA) software was used for performing statistical analysis.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 RP-HPLC method development

The RP-HPLC method was validated for quantitative analyses of phenylethanoids (salidroside and tyrosol) as per the guidelines of ICH [**223**]. As shown in **Table 4.1**, the RT for salidroside and tyrosol was 1.507 ± 0.019 and 2.400 ± 0.02, respectively. The maximum absorbance of both the standards was recorded to be $225\ \text{nm}$. The linear regression equation for salidroside was found to be $y = 8.41\times10^{3}x + 3.13\times10^{3}$, whereas, for tyrosol, the linear regression equation was recorded to be $y = 2.4\times10^{4}\ x + 820.98$. The calibration curves showed good linearity at tested concentrations $(1–625\ \mu g/ml)$ with correlation coefficients (R^2) of 0.99 for both the standards. The LOD and LOQ for salidroside was recorded to be $0.15\ \mu g/ml$ and $0.49\ \mu g/ml$, respectively, whereas, for tyrosol, it was recorded to be $0.10\ \mu g/ml$ and $0.33\ \mu g/ml$, respectively. The intra- and inter-day RSD value of retention time for salidroside was $0.55\ \%$ and $1.25\ \%$, respectively, whereas, for tyrosol, it was found to be $0.42\ \%$ and $0.85\ \%$, respectively. The intra- and inter-day RSD value of peak area for salidroside was $1.40\ \%$ and $2.45\ \%$, respectively, whereas, for tyrosol, it was found to be $1.43\ \%$ and $1.94\ \%$, respectively. The accuracy and accuracy RSD value for salidroside were $99.18\ \%$ and $1.81\ \%$, respectively. The accuracy and accuracy RSD value for tyrosol were $101.42\ \%$ and $1.92\ \%$, respectively. These findings explicitly unveil that RP-HPLC method was rapid accurate, and reproducible for precise quantification of phenylethanoids in processed samples of *R. imbricata.*

Table 4.1: Validated data for quantitative estimation of phenylethanoids (salidroside and tyrosol)

Compound	T_r (min)	λ_{max} (nm)	Regression equation (n = 4)	r^2	Linear range µg/ml	LOD µg/ml	LOQ µg/ml	Precision				Accuracy	
								Retention time		Peak Area			
								Intra (n=15) RSD %	Inter (n=15) RSD %	Intra (n=15) RSD %	Inter (n=15) RSD%	Accuracy (%) (n=15)	RSD % (n=15)
Salidroside	1.507	225	$y = 8.41x10^3x + 3.13x10^3$	0.999	1-625	0.15	0.49	0.55	1.25	1.4	2.45	99.18	1.81
Tyrosol	2.4	225	$y = 2.4x10^4 x + 820.98$	0.999	1-625	0.1	0.33	0.42	0.85	1.43	1.94	101.42	1.92

4.2 Screening and selection of elite chemotypes of *Rhodiola imbricata*

A validated RP-HPLC method was used for analysis of phenylethanoids (salidroside and tyrosol) in natural and field accessions of *R. imbricata*. RP-HPLC analysis showed a significant variability in the composition and content of major phenylethanoids in natural accessions of *R. imbricata* (**Table 4.2**). The maximum amount of salidroside was observed in accessions collected from Khardungla (2.21 ± 0.03 mg/g DW) and Changla (2.22 ± 0.02 mg/g DW) followed by Penzila. (0.79 ± 0.02 mg/g DW), whereas the highest amount of tyrosol was recorded in Khardungla (4.27 ± 0.03 mg/g DW) and Penzila (2.1 ± 0.03 mg/g DW) accessions (**Table 4.2, Figure 4.1 a, b**). However, the tyrosol was absent in Changla accessions. The percentage composition of salidroside and tyrosol in Khardungla accession (KA4) was 34.10 % salidroside and 65.90 % tyrosol, whereas, Penzila accession (PA5) showed 19.56 % salidroside and 80.54 % tyrosol. These variations in major bioactive constituents of *R. imbricata* might be attributed to the diverse fluctuating biotic and abiotic challenges across different geographical locations [**238, 239**]**.** Previous studies have suggested that variations in major bioactive compounds depends on tissue type, seasonal effects and geographical locations [**240, 241**]. Similar phytochemical variations have also been observed in *Rhodiola rosea* [**242**] and *Rhodiola sacchalinensis* [**243**]. These observed phytochemical variations might contribute to the overall fitness of *R. imbricata* in ecologically distinct environment. The results also demonstrate that the amount of salidroside and tyrosol increases with increase in altitude gradient, on an average, the Khardungla accessions showed maximum content of salidroside and tyrosol (1.86 ± 0.02 mg/g DW), followed by Changla (1.08 ± 0.02 mg/g DW) and Penzila (0.57 ± 0.01 mg/g DW) (**Table 4.2**). Wang et al. [**244**] also found that rhubarb grown at higher altitude has higher anthraquinone glycoside content. Similar results have also been reported in other high-altitude medicinal plants [**245, 246**]. In this study, the levels of salidroside and tyrosol also varied spatially, the salidroside content being highest in rhizome (1.7 ± 0.02 mg/g DW) followed by leaf (1.27 ± 0.02 mg/g DW), stem (1.12 ± 0.02 mg/g DW) and root (1.02 ± 0.01 mg/g DW), whereas, the tyrosol content was highest in root (4.27 ± 0.03 mg/g DW) and rhizome (2.1 ± 0.03 mg/g DW) (**Table 4.2**). These phytochemical variations in different plant parts could be explained according to optimal defence theory, which relates these variations to importance of resource allocation to specific

plant organ with their associated biotic interactions [**246**]. Several studies have suggested that the biosynthesis of these secondary metabolites is under tight spatial and temporal modulation of gene expression [**247**]. Similar spatial variations have been observed in *Rhodiola rosea* [**242**].

RP-HPLC analysis showed high variability in the composition and concentration of major phenylethanoids in field accessions of *R. imbricata* (**Table 4.3**). The highest amount of salidroside was observed in accessions collected from Khardungla (1.51 ± 0.02 mg/g DW) followed by Changla (1.28 ± 0.02 mg/g DW) and Penzila (1.20 ± 0.02 mg/g DW), whereas tyrosol was detected under the limit of quantification in Penzila accessions (**Table 4.3, Figure 4.1 c, d, e**). These variations in major phenylethanoids could be genetically controlled. Similar variations have also been observed in field-cultivated *Rhodiola rosea* [**248**].

Therefore, the present findings for the first time reveal significant spatial variations in content of major phenylethanoids (salidroside and tyrosol) in natural and field-accessions of *R. imbricata* collected from different geographical locations of trans-Himalayan Ladakh region. KA4, CA1 and PA5 was identified as an elite chemotypes on the basis of composition and high concentration of major phenylethanoids (salidroside and tyrosol). The identification of elite chemotypes is of considerable significance for strain improvement, obtaining high producing cell lines and to fulfill the stringent requirements of phyto-pharmaceutical industries [**42**].

Table 4.2: Quantitative analysis of phenylethanoids in natural accessions of *Rhodiola imbricata*

Location	ID	Latitude (N)	Longitude (E)	Altitude (m)	Phenylethanoids									
					SALIDROSIDE (mg/g DW)					TYROSOL (mg/g DW)				Total Salidroside + Tyrosol (mg/g DW)
					Leaf	Stem	Rhizome	Root	Total	Leaf	Stem	Rhizome	Root	
Changla	**CA1**	34.255	78.130	5355 ± 6.6	ND*	ND	**1.27±0.02**	**0.95±0.02**	**2.22±0.02**	ND	ND	ND	ND	**2.22±0.02**
Changla	CA2	34.273	78.136	5350 ± 6.6	ND	ND	1.7±0.02	ND	1.7±0.02	ND	ND	ND	ND	1.7±0.02
Changla	CA3	34.255	78.137	5352 ± 5.7	0.98±0.02	ND	DUL^	ND	0.98±0.02	ND	ND	ND	ND	0.98±0.02
Changla	CA4	34.255	78.138	5336 ± 7.2	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Changla	CA5	34.255	78.140	5340 ± 4.6	0.95±0.02	ND	ND	DUL	0.95±0.02	ND	ND	ND	ND	0.95±0.02
Changla	CA6	34.257	78.139	5339 ± 4.6	ND	0.6±0.012	ND	DUL	0.6±0.012	ND	ND	ND	ND	0.6±0.012
Khardungla	KA1	34.289	77.875	5663 ± 9.2	1.27±0.02	ND	ND	ND	1.27±0.02	ND	ND	ND	ND	1.27±0.02
Khardungla	KA2	34.277	77.876	5614 ± 2.8	0.79±0.02	ND	ND	DUL	0.79±0.02	ND	ND	ND	ND	0.79±0.02
Khardungla	KA3	34.292	77.878	5618 ± 3.0	ND	1.12±0.02	0.62±0.02	ND	1.74±0.02	ND	ND	ND	DUL	1.74±0.02
Khardungla	**KA4**	34.293	77.879	5632 ± 6.2	0.5±0.01	ND	**0.69±0.02**	**1.02±0.01**	**2.21±0.03**	ND	ND	ND	**4.27±0.03**	**6.48±0.03**
Khardungla	KA5	34.296	77.880	5638 ± 5.7	ND	ND	DUL	DUL	DUL	ND	ND	ND	ND	DUL
Khardungla	KA6	34.294	77.883	5646 ± 6.1	ND	0.89±0.02	ND	ND	0.89±0.02	ND	ND	ND	ND	0.89±0.02
Penzila	PA1	33.856	76.562	4121 ± 13.3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Penzila	PA2	33.894	76.588	4213 ± 5.9	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Penzila	PA3	33.881	76.575	4354 ± 11.4	DUL	ND	DUL	ND	DUL	ND	ND	ND	ND	DUL

Penzila	PA4	33.959	76.463	4321 ± 9.3	ND	ND	ND	ND	ND	ND	ND	ND	DUL	DUL
Penzila	**PA5**	33.959	76.454	4121 ± 8.9	**0.51±0.01**	ND	DUL	DUL	**0.51±0.01**	ND	ND	**2.1±0.02**	ND	**2.61±0.02**
Penzila	PA6	33.957	76.462	4123 ± 10.9	0.79±0.02	ND	ND	ND	0.79±0.02	ND	ND	ND	ND	0.79±0.02

Values are mean ± standard deviation of three replicates. ND* – Not detected, DUL^ – Detected under the limit of quantification.

Table 4.3: Quantitative analysis of phenylethanoids in field-cultivated accessions of *Rhodiola imbricata*

ID	**Latitude (N)**	**Longitude (E)**	**Altitude (m)**	**PHENYLETHANOIDS**								
				SALIDROSIDE (mg/g DW)					**TYROSOL (mg/g DW)**			
				Leaf	**Stem**	**Rhizome**	**Root**	**Total**	**Leaf**	**Stem**	**Rhizome**	**Root**
CA1	34.255	78.13	5355 ± 6.6	ND*	ND	0.61±0.02	0.67±0.02	1.28±0.02	ND	ND	ND	ND
KA4	34.293	77.879	5632 ± 6.2	ND	ND	0.73±0.02	0.78±0.02	1.51±0.02	ND	ND	ND	ND
PA5	33.959	76.454	4121 ± 8.9	ND	ND	0.61±0.02	0.59±0.02	1.20±0.02	ND	DUL^	ND	ND

Values are mean ± standard deviation of three replicates. ND* – Not detected, DUL^ – Detected under the limit of quantification.

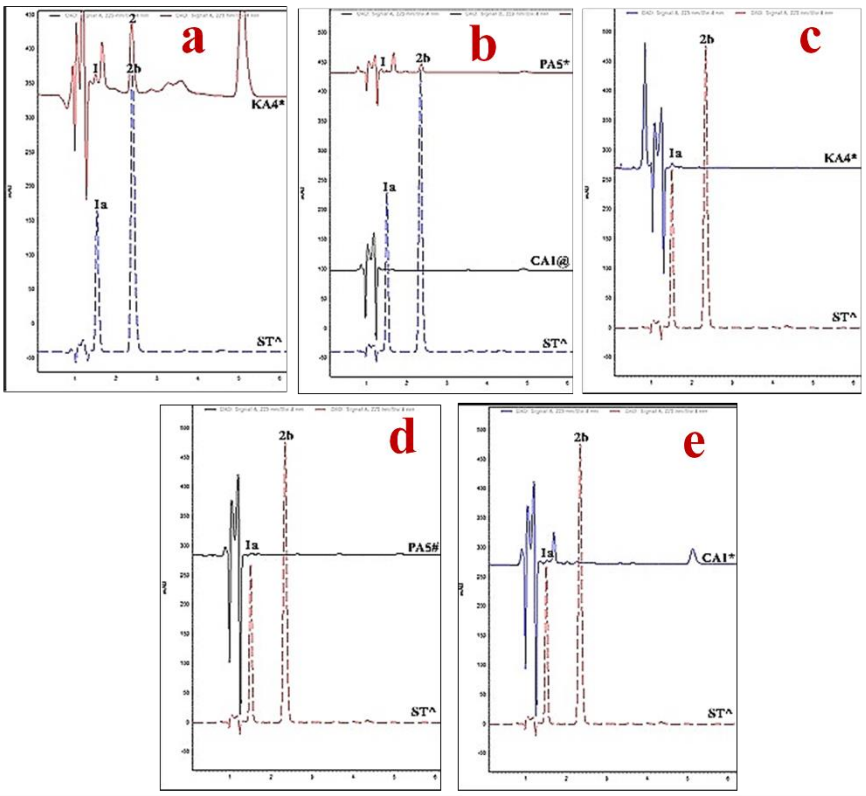


Figure 4.1: HPLC chromatograms of (**a-b**) natural accession and (**c-e**) field accessions. **1a**: Salidroside, **2b**: Tyrosol, **ST**^: Standard, **KA4***: Khardungla A4 accession, **PA5**#: Penzila A5 accession and **CA1**@: CA1 accession.

4.3 Effect of plant growth regulators and explant type on callus induction in *Rhodiola imbricata* chemotypes

Callus culture system represent a promising alternative source of valuable bioactive metabolites [**34**]. Callus cultures have also been widely used in research and industrial applications [**249].** The success of callus induction mainly depends on optimum concentration and combination of PGRs, explant type and plant species [**250**]. Therefore, in this study, the sterilized stem and leaf explant from elite chemotypes (KA4, CA1 and PA5) of *R. imbricata* were cultured on MS medium containing various concentrations of BA (0-5 mg/l) and NAA (0-5 mg/l). Callus was induced from both explants in response to all applied concentrations of PGRs (**Table 4.4**). However, the CIF alter in response to varied concentrations of PGRs. The 5 mg/l NAA + 5 mg/l BA reveal highest percentage of callus induction (93 %), as compared to other concentrations (**Table 4.4**, $p \leq 0.05$). The lower

concentrations of NAA + BA showed minimum percentage of callus induction than the higher concentrations of NAA + BA (**Table 4.4**, $p \leq 0.05$). Khanpour-Ardestani et al. [**251**] also reported that callus was successfully induced from all the combinations of NAA and BA in *Scrophularia striata. Modarres et al.* [**252**] found that MS medium containing 5 mg/l NAA + 5 mg/l BA was suitable for callus induction in *Salvia leriifolia.* The synergistic combinations of NAA and BA also resulted in maximum callogenic frequency in *in-vitro* cultures of *Aquilaria malaccensis* [**253**], *Cynara scolymus* [**254**], *Scrophularia striata* [**251**], *Ecballium elaterium* [**255**] and *Rhodiola crenulata* [**149**]. The exogenously applied PGRs may alters the endogenous synthesis of PGRs, which maintains the ratio that favours callus formation [**256].** The auxin and cytokinin containing medium generally up-regulate the expression of LBD16, LBD17, LBD18, and LBD29 for optimum callus induction [**257**]. The callus induced from higher concentrations of NAA + BA was green and compact, while the callus cultures induced from lower concentrations of NAA + BA was green or yellowish-white and semi-friable in nature (**Figure 4.2**). These morphological variations observed under different PGRs treatments might be attributed to the differences in physiology and biochemistry of explant [**258**]. Similar morphological variations have also been observed under different concentrations and combinations of NAA and BA in callus culture of *Heliotropium indicum* [**259**], *Rhodiola crenulata* [**149**] and *Linum usitatissimum* [**258**]. In this study, the stem and leaf explant respond differentially to different concentrations of PGRs (**Table 4.4**). The leaf explant was more conducive for callus induction, as compared to the stem explant ($p \leq 0.05$). The callus was induced within 10-15 days in leaf explant, whereas in the stem explant the callus was induced in 20 days (**Figure 4.2**). These differences in response to different PGRs concentrations might be attributed to varied amount of endogenous hormones or distinct tissue sensitivity [**260**]. Shi et al. [**149**] also reported that leaf explants showed highest callus induction when compared to stem and root explants of *Rhodiola crenulata.* Khanpour-Ardestani et al. [**251**] found that callus was successfully induced from both the stem and leaf explants of *Scrophularia striata.* The different elite chemotypes (KA4, PA5 and CA1) of *R. imbricata* showed highly variable callus induction frequency. The highest callus induction frequency was recorded in KA4 ($93 \pm 1.2\%$), followed by CA1 ($85 \pm 0.9\%$) and PA5 ($84 \pm 0.9\%$). These variations could be attributed to genetic differences among chemotypes. The results suggest that a particular concentration of PGRs (NAA and BA) and type of explant regulates the callus induction

response in *R. imbricata* chemotypes. The present study demonstrated that MS medium fortified with 5 mg/l NAA and 5 mg/l BA was highly efficacious for callogenesis from leaf explants of KA4 chemotype of *R. imbricata* for the propose of establishing suspension cultures as well as production of precious bioactive metabolites.

Table 4.4: Effect of PGRs and explant type on callus induction in *Rhodiola imbricata* chemotypes

Phytohormones concentrations (mg/l)		Callus Induction Frequency (%) *					
		Leaf explant			Stem explant		
NAA	BAP	CA1	KA4	PA5	CA1	KA4	PA5
0	0	--	--	--	--	--	--
0.1	0.1	40.08±0.2 ^{a}	39.58±0.3^{a}	39.08±0.3^{a}	35.08±0.2 ^{a}	36.58±0.3^{a}	30.08±0.2 ^{a}
0.1	1	41.58±0.2 ^{b}	41.08±0.5^{b}	40.58±0.4 ^{b}	34.58±0.3 ^{a}	37.08±0.2^{a}	32.58±0.3^{b}
0.1	3	51.38±0.3^{c}	50.82±0.5^{c}	50.27±0.6^{c}	46.38±0.3^{b}	47.82±0.4^{b}	41.27±0.4^{c}
0.1	5	53.03±0.8 ^{d}	52.48±0.3^{d}	51.93±0.5^{d}	47.03±0.4 ^{b}	47.48±0.3^{b}	43.93±0.5^{d}
1	0.1	35.59±0.2^{e}	35.09±0.2^{e}	34.60±0.3^{e}	30.59±0.2^{c}	31.09±0.2^{c}	26.60±0.2^{e}
1	1	54.68±0.6^{f}	54.13±0.4^{f}	53.58±0.6^{f}	48.68±0.3^{d}	49.13±0.4^{d}	45.58±0.4^{f}
1	3	56.34±0.5 ^{g}	55.79±0.6^{g}	55.24±0.7 ^{g}	50.34±0.5^{e}	50.79±0.4^{e}	47.24±0.4^{g}
1	5	57.14±0.4^{gh}	59.14±0.5^{h}	56.04±0.6 ^{g}	51.14±0.4^{e}	55.14±0.5^{f}	47.04±0.3^{g}
3	0.1	42.86±0.3 ^{b}	43.00±0.4 ^{i}	41.86±0.5 ^{b}	35.86±0.3 ^{a}	40.00±0.3 ^{g}	34.86±0.2 ^{h}
3	1	49.72±0.4^{i}	49.17±0.3^{j}	48.62±0.3^{h}	45.72±0.4 ^{b}	47.17±0.5^{b}	40.62±0.3 ^{c}
3	3	60.07±0.3^{j}	63.52±0.4^{k}	59.96±0.4^{i}	53.07±0.3^{f}	58.52±0.3^{h}	52.96±0.3^{i}
3	5	76.41±0.3^{k}	79.86±0.5^{l}	75.31±0.5^{j}	72.41±0.2^{g}	74.86±0.4^{i}	70.31±0.4^{j}
5	0.1	46.10±0.2^{l}	48.03±0.4^{m}	49.96±0.4 ^{ch}	42.10±0.4^{h}	43.03±0.2^{j}	41.96±0.5 ^{c}
5	1	51.89±0.3 ^{cd}	53.82±0.5 ^{df}	55.75±0.5 ^{k}	46.89±0.4 ^{b}	49.82±0.3^{de}	46.75±0.2^{g}
5	3	57.68±0.5^{h}	59.61±0.4 ^{h}	61.54±0.6^{l}	53.68±0.4 ^{f}	56.61±0.5^{k}	55.54±0.7^{k}
5	5	**85.00±0.9^{m}**	**93.00±1.2^{n}**	**84.00±0.8^{m}**	**77.00±0.6^{i}**	**80.00±0.7^{l}**	**73.00±0.6^{l}**

Values are mean ± standard deviation of three replicates. Mean with similar letters are not significantly different at $p \leq 0.05$ according to Bonferroni post-hoc test.

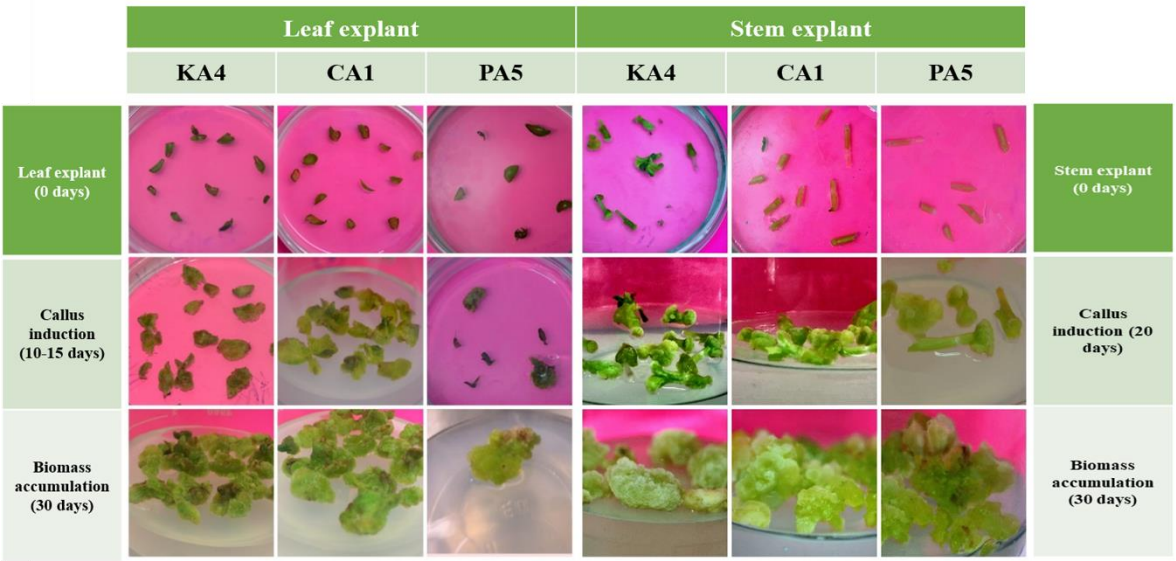


Figure 4.2: Callus induction and biomass accumulation from leaf and stem explants of elite chemotypes of *Rhodiola imbricata.*

4.4 Effect of PGRs and explant type on biomass accumulation in callus cultures of *Rhodiola imbricata* chemotypes

The concentration of PGRs play vital role in biomass accrual in callus cultures [**261**]. The type of explant also markedly affects the biomass accumulation in cell cultures [**262**]. However, the response to different concentrations of PGRs and explant type is species-specific [**263**]. Therefore, in this study, the callus initiated from stem and leaf explants of elite chemotypes (KA4, CA1 and PA5) of *R. imbricata* were inoculated on modified MS medium fortified with various concentrations of BA (0-3 mg/l) and NAA (0-3 mg/l). In case of leaf explant, the maximum amount of biomass (115 ± 5 g/l FW and 7.45 ± 0.5 g/l DW) was observed in response to 3 mg/l NAA + 3 mg/l BA, while the minimum biomass accrual (99.11 ± 5 g/l FW and 6.99 ± 0.5 g/l DW) was observed in response to 0.1 mg/l NAA + 0.1 mg/l BA (**Figure 4.2, Figure 4.3 a, b**). In case of stem explant, the maximum amount of biomass (98 ± 5 g/l FW and 7 ± 0.5 g/l DW) was observed in response to 3 mg/l NAA + 3 mg/l BA, while the minimum amount of biomass (92.11 ± 5 g/l FW and 6.45 ± 0.6 g/l DW) was observed in response to 0.1 mg/l NAA + 0.1 mg/l BA (**Figure 4.2, Figure 4.3 c, d**). These findings clearly demonstrated that the leaf explant showed higher biomass accumulation when compared to the stem explant ($p \leq 0.05$). Similar response of higher biomass accumulation from leaf explant was previously reported in other species [**258**]. These differences in explants response might be due to variability in their genetic constitution [**264**]. The higher concentration of NAA (3 mg/l) + BA (3 mg/l) was found to be more responsive towards biomass accrual in callus of *R. imbricata*. The synergistic effect of NAA and BA on biomass accumulation is also reported in *in-vitro* cultures of *Aronia melanocarpa* **[265],** *Stellera chamaejasme* [**266]** and *Hypericum perforatum* **[261**]. The exogenously applied PGRs generally triggers cell expansion and cell proliferation [**267**]. The auxins exhibit synergistic and antagonistic interactions with cytokinins in regulating physiological responses [**263].** The different elite chemotypes (KA4, PA5 and CA1) of *R. imbricata* showed high variability in biomass accumulation pattern in callus cultures. The maximum biomass accumulation was recorded in KA4 (115 ± 5 g/l FW, 7.45 ± 0.5 g/l DW), followed by CA1 (100.91 ± 6 g/l FW, 7.06 ± 0.5 g/l DW) and PA5 (99.91 ± 5 g/l FW, 6.99 ± 0.6 g/l DW) (**Figure 4.3**). These variations in biomass accumulation in callus cultures could be genetically regulated among different chemotypes.

Callus cultures derived from two different chemotypes of *Scoparia dulcis* also showed varied growth rate under similar physiochemical conditions [**268**]. These findings reveal that a particular concentration of PGRs (NAA and BA) and explant type have important bearings on the biomass accrual in callus of *R. imbricata* chemotypes. In the present study, we have successfully optimized that MS medium containing 3 mg/l NAA and 3 mg/l BA favours highest biomass accumulation in callus cultures derived from leaf explants of KA4 chemotype of *R. imbricata.*

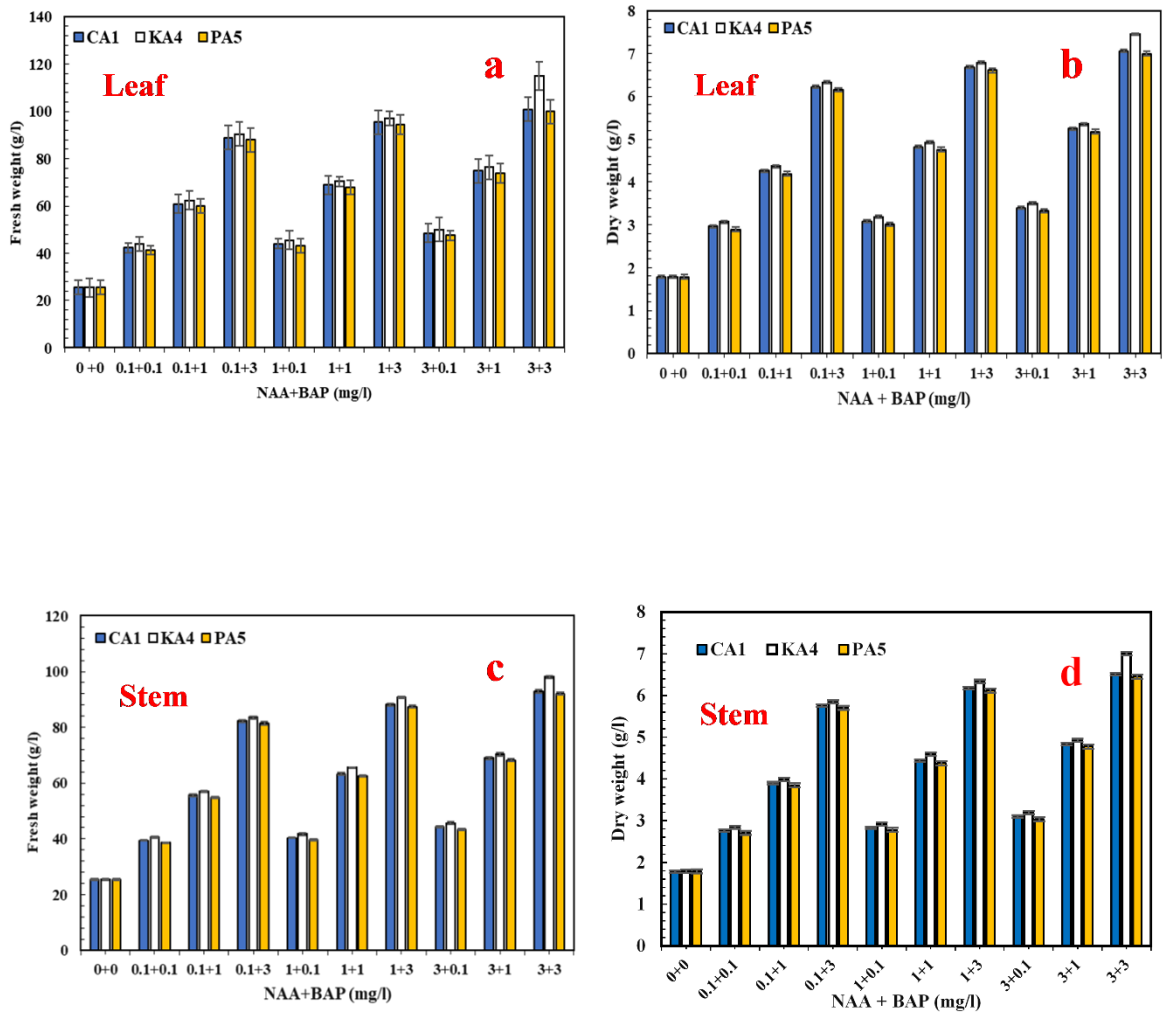


Figure 4.3: Effect of PGRs and explant type on biomass accumulation in callus cultures of *Rhodiola imbricata* chemotypes. Values are mean ± standard deviation (vertical error bars) of three replicates.

4.5 Effect of plant growth regulators on phenylethanoids production in callus cultures of *Rhodiola imbricata* chemotypes

The PGRs markedly influence secondary metabolism in *in-vitro* cultures [**183].** However, the response is immensely contingent on type and concentrations of PGRs and plant species [**183].** Therefore, in this study, we examined the influence of various concentrations of PGRs (BA (0-3 mg/l) + NAA (0-3 mg/l)) on phenylethanoids accrual in callus cultures of *R. imbricata* chemotypes. The 3 mg/l NAA + 3 mg/l BA displayed highest accrual of salidroside (2.62 ± 0.02 mg/g DW), as compared to other concentrations ($p \leq 0.05$, **Figure 4.4**). NAA and BA at lower concentrations lead to a substantial decrease in salidroside accumulation, as compared to higher concentrations ($p \leq 0.05$, **Figure 4.4, Figure 4.5**). The tyrosol was found to be absent in cultures, probably due to their role as precursor for leading to salidroside biosynthesis. The PGRs alters the expression of several enzymes in shikimate pathway for the synthesis of phenolic compounds [**269].** The marked influence of NAA and BA on synthesis of phenolics is also reported in *in-vitro* cultures of *Schisandra chinensis* **[270]**, *Hypericum perforatum* **[261**], *Acaciella angustissima* [**271]** and *Scrophularia striata* [**251].** The exogenously applied PGRs modulates the physiological and biochemical processes through synthesis and degradation of endogenous hormones [**272].** Among different elite chemotypes, KA4 chemotype showed highest salidroside content (2.62 ± 0.02 mg/g DW) on MS medium containing 3 mg/l NAA + 3 mg/l BA (**Figure 4.4, Figure 4.5**). These variations in phenylethanoids production might be due to the genetic differences among the chemotypes. Callus tissues derived from two different chemotypes of *Scoparia dulcis* also showed differential accumulation pattern of scoparic acid A and scopadulcic acid B [**268**]. The authors have attributed these variations to differences in expression pattern of genes encoding key biosynthetic enzymes. Renu et al. [**273**] also found a differential accumulation of phenylpropanoids in calli obtained from eight different chemotypes of *Ocimum tenuiflorum* and attributed these differences to chemotype specific differential gene expression of EOMT. Callus cultures of *R. imbricata* showed 1.23-fold higher content of salidroside, as compared to the parent plant ($p \leq 0.05$). Ouyang et al. [**274**] also found that callus cultures of *Cistanche deserticola* have higher amount of phenylethanoid glycosides when compared to the mother plant, probably due to the stimulation of PEGs synthesis by PGRs. Similar results are also demonstrated in *in-vitro* cultures of *Aronia melanocarpa* [**265**], *Salvia miltiorrhiza* [**275**] and *Ephedra major* [**276**].

These results unveil the differential impact of NAA and BA on salidroside content in callus of *R. imbricata*. Therefore, in the present study, we have successfully optimized the PGRs concentration (NAA (3 mg/l) and BA (3 mg/l)) for substantial synthesis of salidroside in callus cultures of *R. imbricata*. These findings would be of great value for industrial and commercial applications.

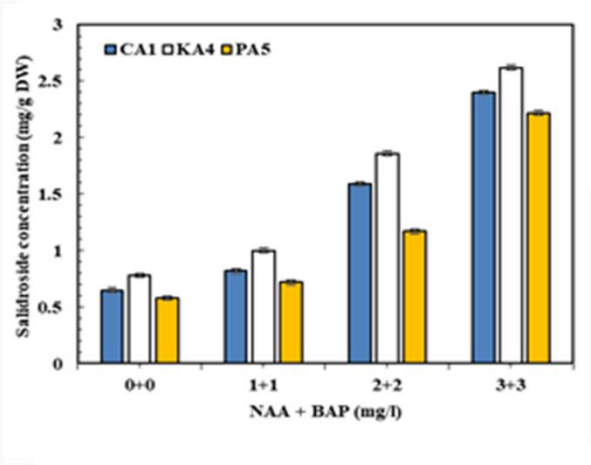


Figure 4.4: Effect of plant growth regulators on phenylethanoids production in callus cultures of *Rhodiola imbricata* chemotypes. Values are mean ± standard deviation (vertical error bars) of three replicates.

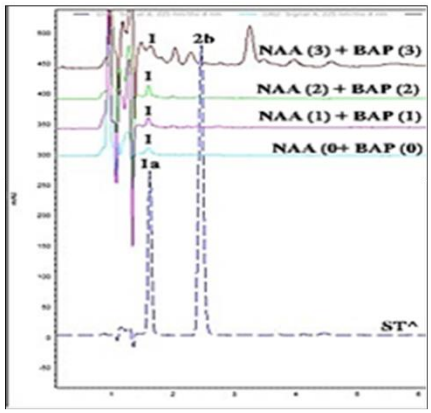


Figure 4.5: HPLC chromatogram illustrating the accumulation of phenylethanoids in callus cultures of *Rhodiola imbricata* cultivated on MS medium containing different concentrations and combinations of NAA and BAP. **1a**: Salidroside, **2b**: Tyrosol and **ST^**: Standard.

4.6 Time-course of biomass accumulation and phenylethanoids production in callus cultures of *Rhodiola imbricata* chemotypes

In this study, we explored the dynamics of biomass and phenylethanoids accumulation in callus cultures derived from leaf explant of different elite chemotypes of *R. imbricata* over a period of 30 days. The callus cultures derived from different elite chemotypes (CA1, KA4 and PA5) of *R. imbricata* showed similar growth and production kinetics (**Figure 4.6 a, b**). The growth curve of calli were characterized by a lag phase (0-5 day), log phase (5-21 day), decelerating phase (21-25 day) and stationary phase (25-30 day). The production curve of callus cultures was characterized by an exponential rise in the accrual of salidroside between day 5 and day 21, followed by a moderate decline until day 30. The callus cultures derived from KA4 chemotype displayed maximum amount of biomass (6.75 ± 0.25 g/l DW) and

maximum salidroside content (2.72 ± 0.02 mg/g DW) on day 21, as compared to other chemotypes (Figure 4.6, $p \leq 0.05$). Similarly, Cheng et al. [**277**] also reported that the maximum biomass accumulation and highest phenylethanoid glycoside production was obtained at day 21 in *in-vitro* cultures of *Cistanche deserticola*. The cultures derived from KA4 chemotype also displayed maximum SGR ($\mu = 0.115$ days^{-1}), DT (144.66 h), GI (2.91) and salidroside yield (18.26 mg/l), as compared to other chemotypes (**Figure 4.6 c, d, e, f,** $p \leq 0.05$). The salidroside production was found to be growth-assocaited in callus cultures. Zhao et al. [**278]** also found that the jaceosidin production was growth-dependent in callus cultures of *Saussurea medusa.* The results suggest that 21st day is ideal harvest time for obtaining maximum biomass yield and salidroside productivity from callus cultures of *R. imbricata.*

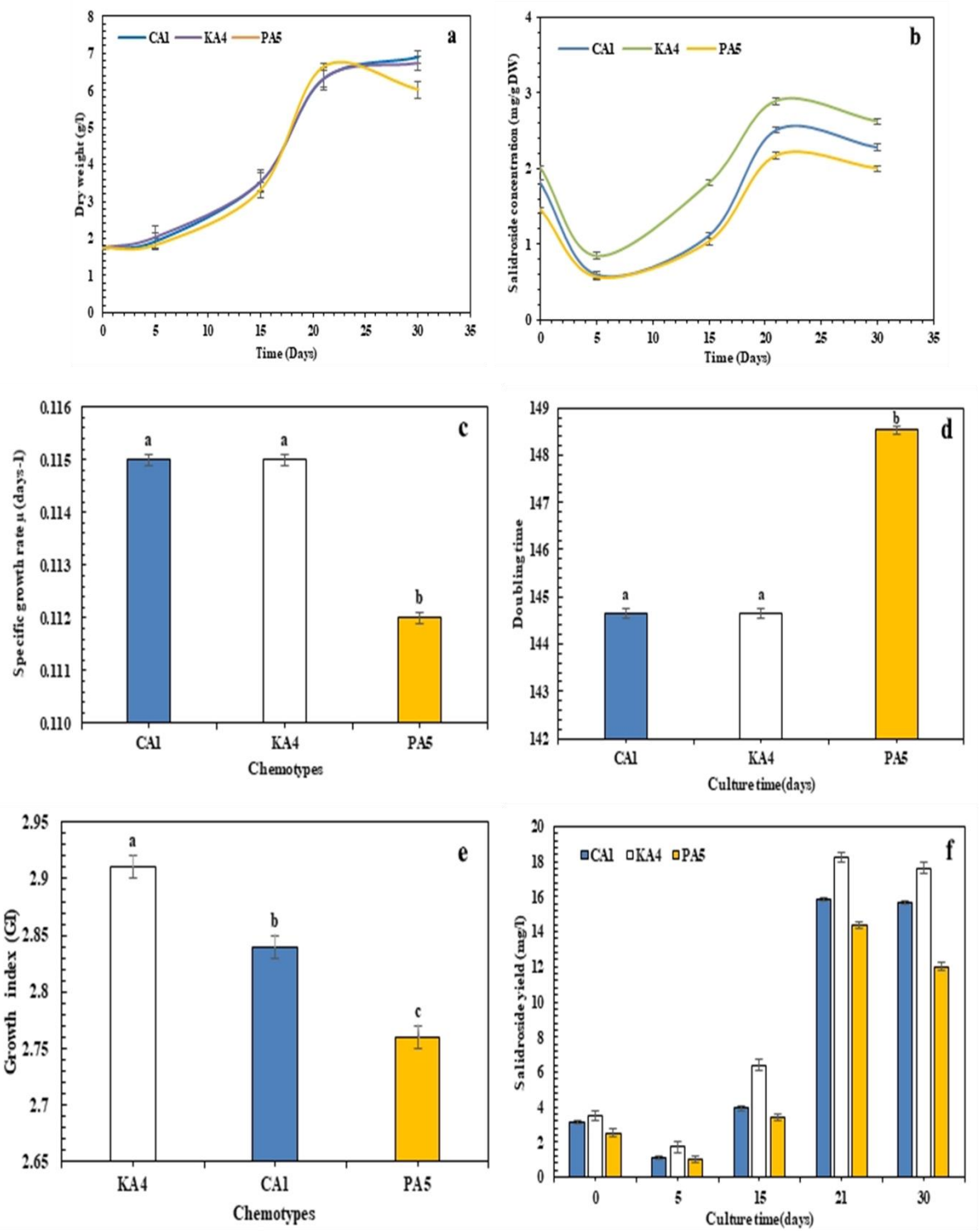


Figure 4.6: (**a**) Dry weight accumulation, (**b**) salidroside accumulation, (**c**) doubling time, (**d**) growth index, (**e**) specific growth rate and (**f**) salidroside yield in callus cultures of *Rhodiola imbricata*. Values are mean ± standard deviation (vertical error bars) of three replicates.

4.7 Analysis of non-enzymatic antioxidants in callus cultures of *Rhodiola imbricata* chemotypes

Plants have evolved an elaborate system of enzymatic (superoxide dismutase, monodehydroascorbate reductase, glutathione reductase, glutathione-S- transferase, ascorbate peroxidase and catalase) and non-enzymatic antioxidants (carotenoids, ascorbate, phenolics, glutathione and α-tocopherol) to keep ROS at a basal non-toxic level [2**79, 280].** The cell cultures of *R. imbricata* have never been explored for the production of non-enzymatic antioxidants. Therefore, the present study investigated the production of non-enzymatic antioxidants (phenolics, flavonoids, flavonol, carotenoids and ascorbate) in callus cultures derived from leaf explants of CA1, KA4 and PA5 chemotypes. As shown in **Figure 4.7,** the TPC (7.08 ± 0.2 mg CHA/g DW), TFC (1.98 ± 0.2 mg RE/g DW), AAC (10.45 ± 0.4 mg/g DW), flavonol (0.98 ± 0.02 mg QE/g DW) and carotenoids (1.6 ± 0.04 mg/g DW) was observed to be significantly higher in callus cultures obtained from KA4 chemotype, as compared to other chemotypes ($p \leq 0.05$). These differences in levels of non-enzymatic antioxidants could be attributed to genetic variations among different chemotypes of *R. imbricata*. These findings revealed for the first time the co-presence of phenolics, flavonoids, flavonol, carotenoids and ascorbic acid in callus of *R. imbricata*. These non-enzymatic antioxidants have numerous health benefits [**281**]**.**

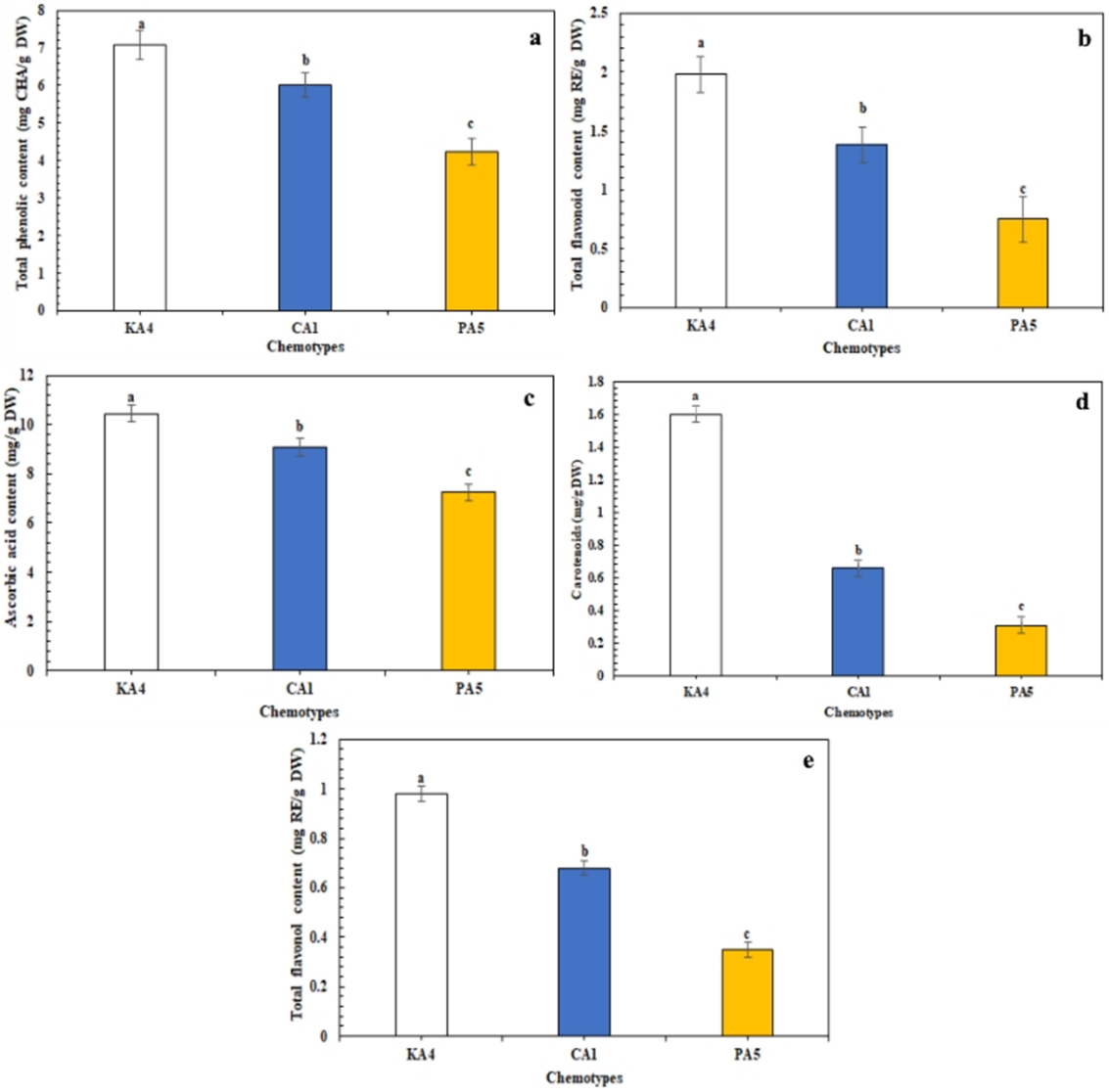


Figure 4.7: Analysis of non-enzymatic antioxidants: (**a**) TPC, (**b**) TFC, (**c**) AAC, (**d**) Carotenoids and (**e**) TFLC in callus cultures of *Rhodiola imbricata* chemotypes. Values are mean ± standard deviation (vertical error bars) of three replicates. Mean with similar letters are not significantly different at $p \leq 0.05$ according to Bonferroni post-hoc test.

4.8 GC-MS analysis of callus cultures of *R. imbricata*

The present study was aimed to investigate the chemical composition of callus of *R. imbricata* by GC-MS. The efficiency of extraction of different phytochemicals depends on several factors, including the method of extraction, matrix composition, solute to solvent ratio, and solvent polarity [**282**]. The choice of a proper solvent is vital for the extraction of bioactive compounds [**283**]. Therefore, the callus cultures were sequentially extracted in solvents with increasing polarity *viz.* hexane, chloroform, ethyl acetate and methanol and subjected to gas chromatographic-mass spectrometric analysis. The n-hexane callus extract

demonstrated the occurrence of 42 distinct phytochemicals (**Table 4.5, Figure 4.8 a**). Among the identified phytochemicals, Stigmast-5-en-3-ol (12.01%), Hexatriacontane (10.65%), Stigmast-4-en-3-one (5.04%), Octacosyl Acetate (4.82%), Tetradecyl acrylate (4.68%), Ergost-5-en-3-ol (3.36%), Tetracosane (2.88%) were in major amount. The chloroform callus extract demonstrated the occurrence of 42 distinct phytochemicals (**Table 4.6, Figure 4.8 b**). Among the identified phytochemicals, Phenol,2,4-Bis(1,1-Dimethylethyl) (11.59%), Benzene,1,3-Bis(1,1-Dimethylethyl) (9.35%), 5-Isobutylnonane (4.65%), 8-Methylheptadecane (4.53%), Tetradecane (2.73%), Hexane, 2,3,3-trimethyl (2.42%), Tetradecane,5-methyl (2.23%), Dotriacontane (2.08%) were in major amount. The ethyl acetate callus extract demonstrated the occurrence of 38 diverse phytochemicals (**Table 4.7, Figure 4.8 c**). Among the identified phytochemicals, Benzene, 1,3-Bis(1,1-Dimethylethyl) (6.44%), Phenol,2,4-Bis(1,1-Dimethylethyl) (6.33%), Tetracontane (4.92%), 5-(2-methylpropyl)nonane (4.24%), 8-methylheptadecane (3.8%), gamma-Sitosterol (3.08%), Eicosane (2.61%), Hexatriacontane (2.58%), Tetrapentacontane (2.56%), Hexane,2,3,3-trimethyl (2.47%), Tetradecane (2.31%), were in major amount. The methanol callus extract demonstrated the occurrence of 20 distinct phytochemicals (**Table 4.8, Figure 4.8 d**). Among the identified phytochemicals, Propane-1,1,3-triol (9.44%), Phenol,2,4-bis(1,1-dimethylethyl) (4.35%), Methyl palmitate (2.48%), Methyl carbamate (2.09%) were in major amount. Therefore, the phytosterols, terpenoids, phenolics, fatty acid ester, alkanes, alkenes and alcohols were identified as major class of phytochemicals in callus extracts of *R. imbricata.*

The major phytochemicals identified in n-hexane, chloroform, and ethyl acetate callus extracts was alkane (n-hexane: 16%, chloroform: 70%, ethyl acetate: 40%). Whereas, fatty acid ester (20%) was the major phytochemical in methanol extract (**Figure 4.9**). The phytochemicals extracted in different solvents showed the following distribution order: alkane (38%), unknown (24%), benzoid (6%), alcohol (4%), phenol (4%), ester (4%), phytosterol (4%), fatty acid ester (3%), ketone (3%), acid ester (3%), alkene (1%), alkaloid (1%) and terpenoids (1%). The sequence of extraction of distinct phytochemicals in different solvents was as follows: (**i**). Alkane: chloroform (70%), ethyl acetate (42%), n-hexane (16%) and methanol (10%), (**ii**). Alkene: chloroform (2%) and ethyl acetate (2%), (**iii**). Alcohol: methanol (10%), chloroform (5%), ethyl acetate (3%) and n-hexane (2%), (**iv**). Benzoid: methanol (15%), n-hexane (7%), chloroform (5%) and ethyl acetate (3%), (**v**). Phenol: n-hexane (7%), methanol (5%), ethyl acetate (3%) and chloroform (2%), (**vi**). Unknown: ethyl

acetate (42%), n-hexane (33%), methanol (5%) and chloroform (2%), (**vii**). Ester: n-hexane (12%) and chloroform (2%), (**viii**). Phytosterol: n-hexane (7%), chloroform (5%) and ethyl acetate (3%), (**ix**). Terpenoids: n-hexane (2%), (**x**). Ketone: methanol (10%), ethyl acetate (3%) and n-hexane (3%), (**xi**). Acid ester: methanol (15%) and n-hexane (2%), (**xii**). Fatty acid ester: methanol (20%) and n-hexane (2%), (**xiii**). Phytol: n-hexane (2%), (**xiv**). Sugar: n-hexane (2%), (**xv**). Ether: ethyl acetate (3%) and (**xvi**). Alkaloid: methanol (10%).

Overall, the GC-MS analysis revealed the presence of 71 distinct phytochemicals in different callus extracts of *R. imbricata* (**Figure 4.10**). The phytochemical database (Dr. Duke Phytochemical and Ethnobotanical database) search demonstrated that the callus extracts possess diverse biological activities, including analgesic, antiarrhythmic, anti-asthmatic, antibacterial, anti-dysenteric, anti-inflammatory, antimutagenic and antioxidant activities (**Table 4.9**), which could be primarily due to the presence of diverse phenolics, terpenoids and fatty acid esters in different callus extracts (**Figure 4.9**). The most of these phytochemicals that have been identified in callus extracts, has also been previously identified in different root extract of *R. imbricata* [**19**], thereby suggesting that the callus of *R. imbricata* is a prospective source of bioactive compounds compared to harvest-based approach. These findings unravel the complex chemical composition, biological activities and therapeutic potential of callus of *R. imbricata*. The characterization and structural elucidation of unidentified compounds may lead to the discovery of novel drugs.

Table 4.5: Phytochemicals identified in n-hexane callus extracts of *Rhodiola imbricata*

S. No	Peak RT (min)	Peak Area	Compound detected
1	3.391	18106	Toluene
2	3.804	78442	2-Hexanol, 3,4-dimethyl
3	4.514	94580	Diacetone alcohol
4	7.293	44608	Decane
5	7.646	119593	alpha- terpinene
6	8.269	84030	5-Butylnonane
7	9.058	87410	Pentanoic Acid, 3-hydroxy-4-methyl-, methyl Ester
8	11.556	118830	Benzene,1,3-Bis(1,1-dimethylethyl)-
9	11.913	64084	Hexadecane,2,6,10,14-tetramethyl-
10	13.352	131374	trans-2-hexenyl butyrate
11	14.827	61274	1-dodecanol
12	15.054	59441	Unknown
13	15.309	112821	Phenol, 2,4-Bis(1,1-dimethylethyl)-
14	16.455	63638	Eicosane
15	16.748	137834	Methyl beta-D-glucopyranoside
16	17.599	563391	Tetradecyl acrylate
17	18.274	10584	Unknown
18	18.607	100312	Ethanone,1-(2,6-Dihydroxy-4-methoxyphenyl)-
19	19.275	79275	Neophytadiene
20	19.341	76831	2-Pentadecanone,6,10,14-trimethyl-
21	19.725	174818	Esculetin
22	20.67	134991	Benzothiazole,2-(2-hydroxyethylthio)-
23	20.955	55461	Ethyl palmitate
24	22.662	68354	Ethyl (9z,12z)-9,12-octadecadienoate
25	23.09	100603	Heptacosyl Acetate
26	25.636	163677	Unknown
27	25.705	346945	Tetracosane
28	26.014	75789	Unknown
29	27.14	159174	Unknown
30	27.193	177698	Unknown
31	27.331	1280827	Hexatriacontane
32	28.276	579573	Octacosyl Acetate
33	28.355	90461	Squalene
34	29.235	98415	Unknown
35	31.515	130969	Unknown
36	31.803	101635	Unknown
37	32.062	134945	alpha-Tocopherol-beta-D-mannoside
38	33.092	51068	Docosyl acetate
39	33.903	404583	Ergost-5-en-3-ol
40	35.499	1444759	Stigmast-5-en-3-ol
41	38.332	605818	Stigmast-4-en-3-one
42	38.613	188367	Unknown

Table 4.6: Phytochemicals identified in chloroform callus extracts of *Rhodiola imbricata*

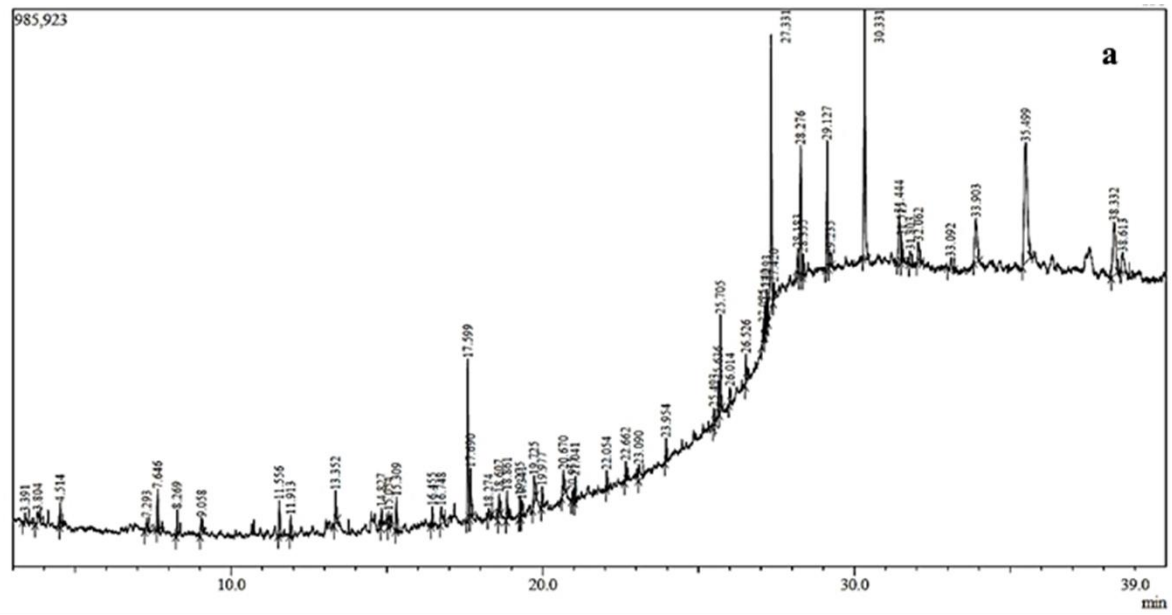
S. No	Peak RT (min)	Peak Area	Peak Area (%)	Compound detected
1	3.215	169797	0.37	Hexane, 3,3-Dimethyl
2	3.306	253276	0.55	Hexane, 2,5-Dimethyl
3	3.741	236356	0.52	1,5-Heptadiene, 3,4-Dimethyl
4	3.787	255616	0.56	Octane
5	4.005	148648	0.32	2-Hexanol, 3,4-Dimethyl
6	4.115	1106993	2.42	Hexane, 2,3,3-Trimethyl
7	4.817	246780	0.54	Octane, 4-Methyl
8	6.638	242774	0.53	Nonane,2-Methyl
9	6.828	201155	0.44	Nonane,2,5-Dimethyl
10	7.29	643988	1.41	Decane
11	7.685	814816	1.78	Decane,4-Methyl
12	8.271	2131976	4.65	Nonane,5-(2-Methylpropyl)
13	10.663	900452	1.97	Azulene
14	10.743	1250485	2.73	Tetradecane
15	10.885	209059	0.46	Sulfurous Acid, Hexyl Nonyl Ester
16	10.943	403444	0.88	Undecane,2,5-Dimethyl
17	11.083	349251	0.76	Dodecane,4-Methyl
18	11.383	323148	0.71	Hexadecane
19	11.56	4282522	9.35	Benzene,1,3-Bis(1,1-Dimethylethyl)
20	11.716	1020506	2.23	Tetradecane,5-Methyl
21	11.917	2076876	4.53	Heptadecane,8-Methyl
22	12.048	389532	0.85	Nonane,5-Methyl-5-Propyl
23	12.769	395012	0.86	4-Nonanol,2,6,8-Trimethyl
24	13.248	169069	0.37	Octadecane
25	13.926	548469	1.2	7,7-Diethylheptadecane
26	13.993	322655	0.7	Eicosane
27	14.065	185485	0.4	4-Methyltetradecane
28	14.61	438859	0.96	Pentadecane,2,6,10,14-Tetramethyl
29	14.65	331812	0.72	2,4-Dimethylicosane
30	15.31	5309745	11.59	Phenol,2,4-Bis(1,1-Dimethylethyl)
31	16.793	210905	0.46	Hexadecane,2,6,10,14-Tetramethyl
32	17.745	224143	0.49	5,5-Diethyltridecane
33	18.211	318461	0.7	Tetracosane
34	19.28	204238	0.45	2-Methylhexacosane
35	20.102	954003	2.08	Dotriacontane
36	24.255	697423	1.52	Tetrapentacontane
37	24.329	369920	0.81	Unknown
38	27.557	459058	1	Unknown
39	30.337	233747	0.51	Octacosyl Acetate
40	34.387	179276	0.39	Unknown
41	35.509	881916	1.93	Stigmast-5-en-3-ol
42	38.351	282540	0.62	Stigmast-4-En-3-One

Table 4.7: Phytochemicals identified in ethyl acetate callus extracts of *Rhodiola imbricata*

S. No	Peak RT (min)	Peak Area	Compound detected
1	4.086	145869	Hexane,2,3,3-trimethyl-
2	7.279	104778	Decane
3	7.675	110254	4-methyldecane
4	8.264	279311	5-(2-methylpropyl) nonane
5	10.739	184534	Tetradecane
6	11.554	470090	Benzene,1,3-Bis(1,1-Dimethylethyl)
7	11.711	80059	5-methyltetradecane
8	11.911	262412	8-methylheptadecane
9	12.615	84785	5-methyl-5-propylnonane
10	14.608	102526	Unknown
11	14.988	225673	Eicosane
12	15.303	471813	Phenol,2,4-Bis(1,1-Dimethylethyl)
13	20.096	116954	Tetracosane
14	20.145	93371	Unknown
15	21.895	76237	Unknown
16	22.054	81659	Octadecane
17	22.271	87187	Hexacontane
18	24.242	69347	Unknown
19	25.63	70689	Unknown
20	25.698	229330	Hexatriacontane
21	25.795	63488	Unknown
22	26.595	83256	Unknown
23	26.685	70486	1,2-Epoxycholestan-3-one
24	26.83	97871	1,2-epoxyoctadecane
25	27.57	69681	Unknown
26	28.174	86852	Dotriaacontane
27	28.568	145719	Cycloheptadecanol
28	29.122	504190	Tetracontane
29	30.747	203308	Hexadecanal
30	31.426	372420	Tetrapentacontane
31	31.815	150482	Unknown
32	32.075	118472	Vitamin E
33	33.722	70753	Unknown
34	33.904	231937	Unknown
35	33.93	84669	Unknown
36	35.486	848325	gamma-Sitosterol
37	38.329	272211	Stigmast-4-en-3-one
38	38.594	139826	Unknown

Table 4.8: Phytochemicals identified in methanol callus extracts of *Rhodiola imbricata*

S. No	Peak RT (min)	Peak Area	Compound detected
1	3.222	1646902	Propane-1,1,3-triol
2	3.306	256841	2-Hydroxyisobutyric acid
3	3.403	679499	Methyl carbamate
4	3.505	115585	Ethyl 4-hydroxyphenylacetate
5	3.596	146298	Unknown
6	4.647	222074	1,1-Diisopropoxypropane
7	7.245	139850	Decane
8	14.335	431711	1,4-Diacetylbenzene
9	14.494	134123	Quinoline, 1,2-dihydro-2,2,4-trimethyl-
10	14.827	142919	1-dodecanol
11	14.931	263485	Acetophenone,4'-(1-hydroxy-1-methylethyl)-
12	15.304	674110	Phenol,2,4-bis(1,1-dimethylethyl)-
13	19.59	205904	Diisobutyl phthalate
14	20.24	407791	Methyl palmitate
15	20.629	283499	Dibutyl phthalate
16	22.002	265513	9,12-Octadecadienoic acid, (z,z)-, methyl ester
17	22.061	136704	Methyl hexadeca-9,12- dienoate
18	22.309	139178	Methyl stearate
19	26.012	186973	Bis(2-ethylhexyl) phthalate
20	29.361	237116	6-(3-fluorobenzylamino) purine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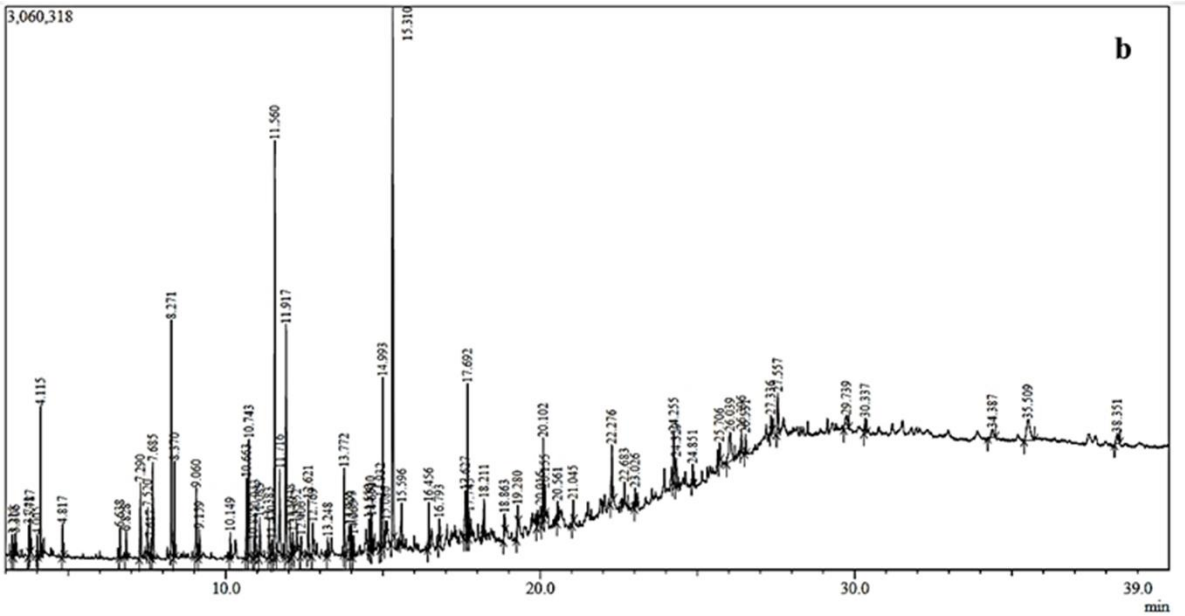
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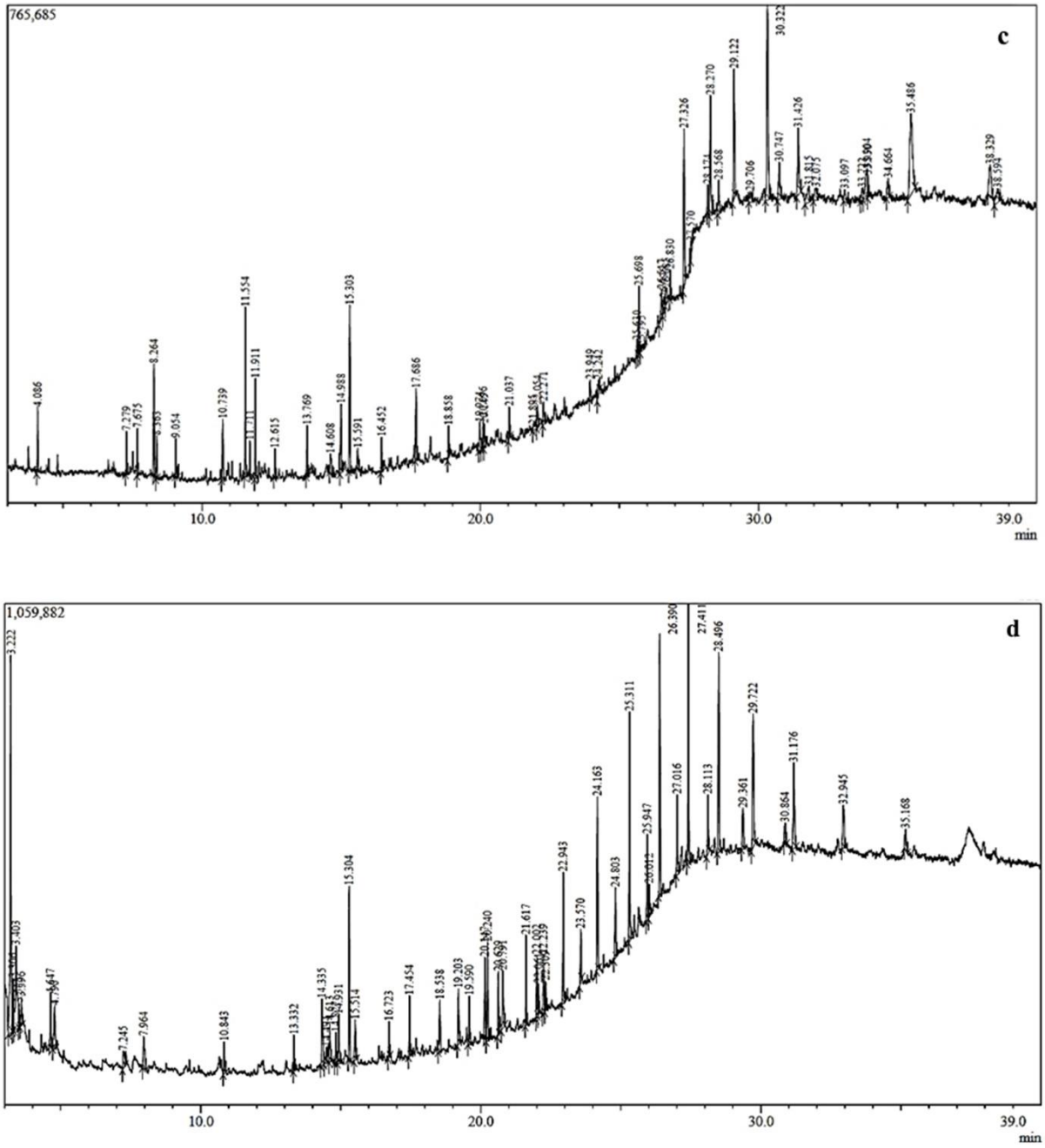


Figure 4.8: Gas chromatograms of (a) n-hexane, (b) chloroform, (c) ethyl acetate and (d) methanol callus extracts of *Rhodiola imbricata.*

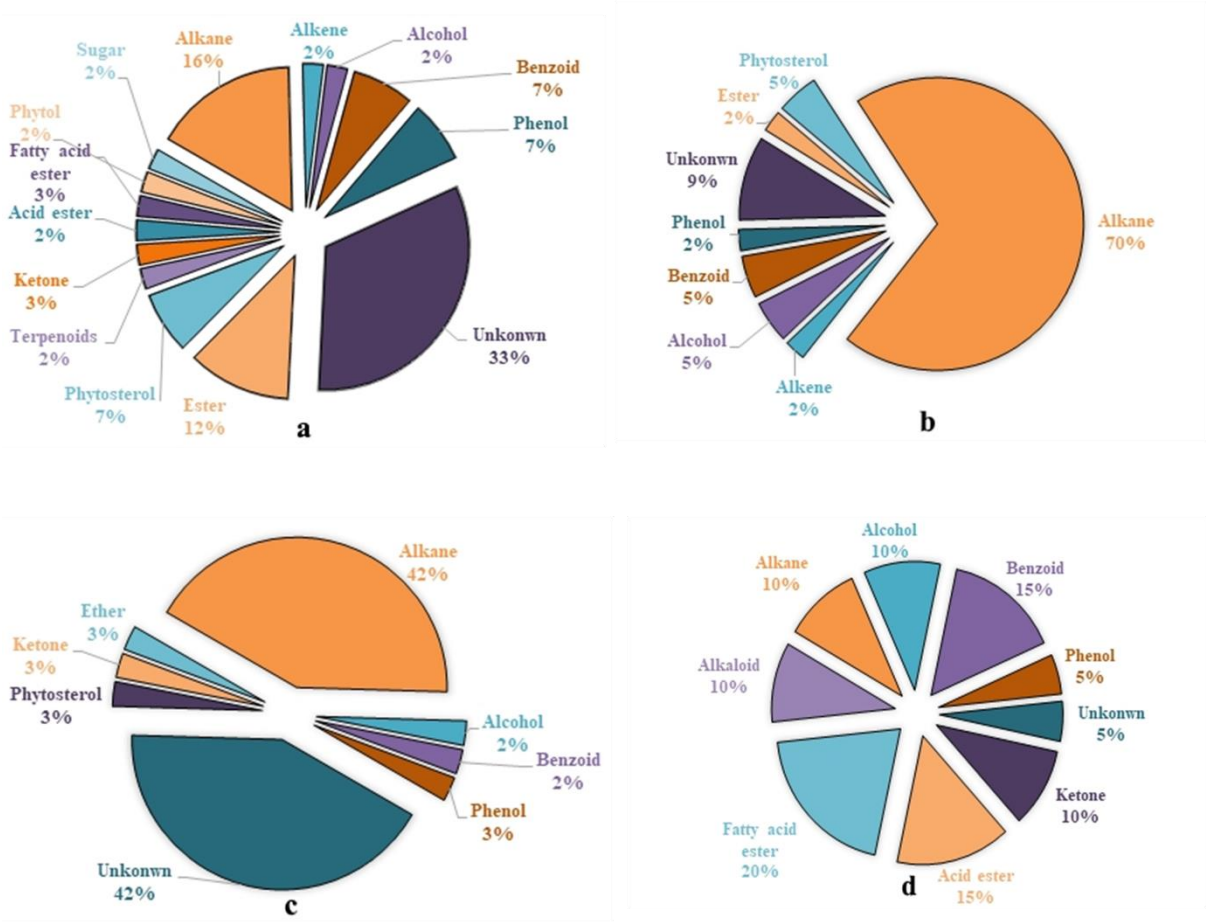
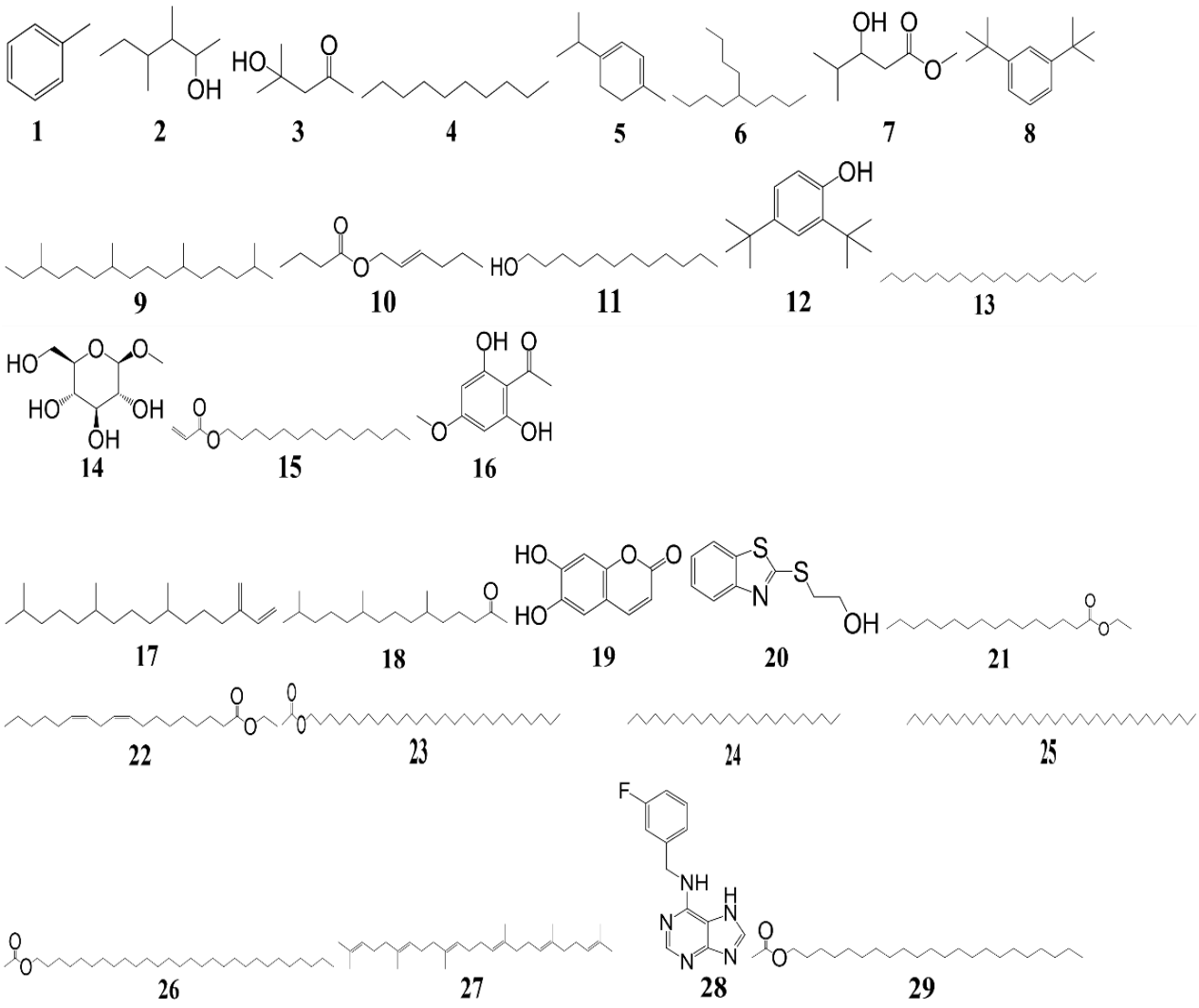


Figure 4.9: Estimation of phytochemical groups in different callus extracts of *Rhodiola imbricata*; (a) n-hexane extract, (b) chloroform extract, (c) ethyl acetate extract and (d) methanol extract.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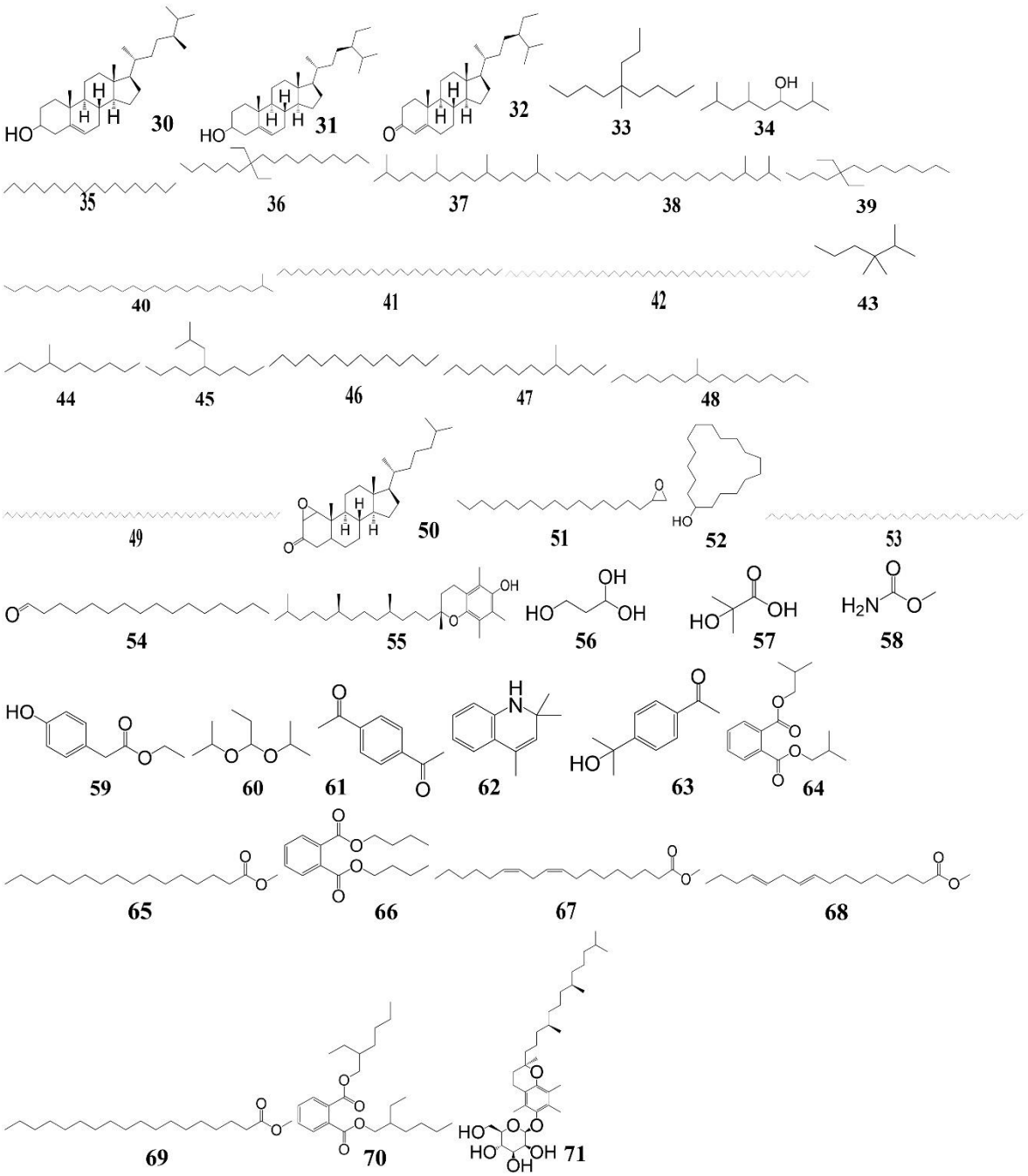

Figure 4.10: Phytochemicals identified in callus extracts of *Rhodiola imbricata,* **1:** Toluene **; 2 :** 2-Hexanol, 3,4-dimethyl **; 3 :** cycloheptadecanol **; 4 :** Decane **; 5 :** alpha- terpinene **; 6 :** 5-butylnonane **; 7 :** Pentanoic Acid, 3-hydroxy-4-methyl-, methyl Ester **; 8 :** Benzene,1,3-Bis(1,1-dimethylethyl)- **; 9 :** Hexadecane,2,6,10,14-tetramethyl- **; 10 :** trans-2-Hexenyl butyrate**; 11 :** 1-dodecanol **; 12 :** Phenol, 2,4-Bis(1,1-dimethylethyl)- **; 13 :** Eicosane **; 14 :** Methyl beta-D-glucopyranoside **; 15 :** Tetradecyl acrylate **; 16 :** Ethanone,1-(2,6-Dihydroxy-4-methoxyphenyl)- **; 17 :** Neophytadiene **; 18 :** 2-Pentadecanone,6,10,14-trimethyl- **; 19 :** Esculetin **; 20 :**

Benzothiazole,2-(2-hydroxyethylthio)- ; **21** : Ethyl palmitate ; **22** : Ethyl (9z,12z)-9,12-octadecadienoate ; **23** : Heptacosyl Acetate ; **24** : Tetracosane ; **25** : Hexatriacontane ; **26** : Octacosyl Acetate ; **27** : Squalene ; **28** : 6-(3-fluorobenzylamino) purine ; **29** : Docosyl acetate ; **30** : Ergost-5-en-3-ol ; **31** : Stigmast-5-en-3-ol ; **32** : Stigmast-4-en-3-one ; **33** : 5-Methyl-5-propylnonane ; **34** : 4-nonanol,2,6,8-trimethyl ; **35** : Octadecane ; **36** : 7,7-Diethylheptadecane ; **37** : Pentadecane,2,6,10,14-tetramethyl ; **38** : 2,4-Dimethylicosane ; **39** : 5,5-Diethyltridecane ; **40** : 2-methylhexacosane ; **41** : Dotriacontane ; **42** : Tetrapentacontane ; **43** : Hexane,2,3,3-trimethyl- ; **44** : 4-methyldecane ; **45** : 5-(2-methylpropyl)nonane ; **46** : Tetradecane ; **47** : 5-methyltetradecane ; **48** : 8-methylheptadecane ; **49** : Hexacontane ; **50** : 1,2-Epoxycholestan-3-one ; **51** : 1,2-epoxyoctadecane ; **52** : Cycloheptadecanol ; **53** : Tetracontane ; **54** : Hexadecanal ; **55** : Vitamin E ; **56** : Propane-1,1,3-triol ; **57** : 2-Hydroxyisobutyric acid ; **58** : Methyl carbamate ; **59** : Ethyl 4-hydroxyphenylacetate ; **60** : 1,1-Diisopropoxypropane ; **61** : 1,4-Diacetylbenzene ; **62** : Quinoline, 1,2-dihydro-2,2,4-trimethyl- ; **63** : Acetophenone,4'-(1-hydroxy-1-methylethyl)- ; **64** : Diisobutyl phthalate ; **65** : Methyl palmitate ; **66** : Dibutyl phthalate ; **67** : 9,12-Octadecadienoic acid,(z,z)-, methyl ester ; **68** : Methyl hexadeca-9,12- dienoate ; **69** : Methyl stearate ; **70** : Bis(2-ethylhexyl) phthalate ; **71** : alpha-Tocopherol-beta-D-mannoside.

Table 4.9: Bioactivities of some phytochemicals in different callus extracts of *Rhodiola imbricata*

Phytochemicals	Bioactivity	Reference
Vitamin E	5-HETE-Inhibitor, Allergenic, Analgesic, Antiaggregant, Antiaging, Antialzheimeran, Antianginal, Antiarteriosclerotic, Antiatherosclerotic, Antibronchitic, Anticariogenic, Anticataract, Antichorea, Anticoronary, Antidecubitic, Antidermatitic, Antidiabetic, Antidysmenorrheic, Antiepitheleomic, Antifibrositic, Antiherpetic, Antiinflammatory, Antiischaemic, Antileukemic, Antileukotriene, Antilithic, Antilupus, Antimastalgic, AntiMD, AntiMS, Antimyoclonic, Antineuritic, Antinitrosaminic, Antiophthalmic, Antiosteoarthritic, Antioxidant, Antiparkinsonian, AntiPMS, Antiproliferant, Antiradicular, Antiretinopathic, Antisenility, Antisickling, Antispasmodic, Antisterility, Antistroke, Antisunburn, Antisyndrome-X, Antithalassemic, Antithrombotic, Antithromboxane, Antitoxemic, Antitumor (Breast), Antitumor (Colorectal), Antitumor (Prostate), Antiulcerogenic, Apoptotic, Hepatoprotective, Hypocholesterolemic, Hypoglycemic, Immunostimulant, Lipoxygenase-Inhibitor, Ornithine-Decarboxylase-Inhibitor, P21-Inducer, Phospholipase-A2-Inhibitor, Protein-Kinase-C-Inhibitor and Vasodilator.	Dr. Duke Phytochemical and Ethnobotanical database (https://phytochem.nal.usda.gov/phytochem)
Alpha-terpinene (Terpenoids)	Acaricide, ACE-Inhibitor, Aldose-Reductase-Inhibitor, Antiacetylcholinesterase, Antinitrosaminic, Antispasmodic, Insecticide, Insectifuge, P450-2B1-Inhibitor, Pesticide and Spasmogenic.	
Esculetin (Phenolic)	5-Lipoxygenase-Inhibitor, Analgesic, Antiarrhythmic, Antiasthmatic, Antibacterial, Anticapillary-Fragility, Antidysenteric, Antiescherichic, Anti-inflammatory, Antimutagenic, Antipyretic, Antiseptic, Antistaphylococcic, Cancer-Preventive, Cardiodepressant, Choleretic, Cosmetic, Cytotoxic, Dermatitigenic, Fungicide, Hepatoprotective, Hypertensive, Lipoxygenase-Inhibitor, Musculotropic, Myorelaxant, Xanthine-Oxidase-Inhibitor, Sunscreen and UV-screen.	
Squalene (Terpenoids)	Antibacterial, Antioxidant, Antitumor, Cancer-Preventive, Chemopreventive, immunostimulant, Lipoxygenase-Inhibitor, Perfumery, Pesticide and Sunscreen.	
Stigmast-4-en-3-one (Phenolics)	Antiprostatitic	

4.9 Effect of basal media on biomass accumulation and phenylethanoids production in CCA suspension cultures of *R. imbricata*

The composition of the basal medium is a vital factor for growth and secondary metabolism in *in-vitro* cultures, as it regulates the gene expression, enzyme activity and carbon flux between primary and secondary metabolites [**284].** The nutritional requirements for optimum growth and secondary metabolites production vary with each plant species [**285].** In this study, the full strength MS media displayed maximum salidroside content $(3.37 \pm 0.03$ mg/g DW$)$ and highest growth (FW: 221.59 ± 0.71 g/l, DW: 8.43 ± 0.01 g/l) in cultures, as compared to other basal media (**Figure 4.11, Figure 4.12,** $p \leq 0.05$). These findings suggest that biomass and salidroside content in cell cultures of *R. imbricata* was distinctly dependent on the basal media. The improved growth and salidroside production in MS medium could be attributed to the high content of nitrate, potassium, and ammonium than B5 media [**284].** Cui et al. [**286**] found that the higher level of nitrate induces high oxidative stress that improved the amount of bioactive molecules in *in-vitro* cultures of *Hypericum perforatum.* The stimulatory influence of MS media is also reported in other *in-vitro* cultures [**39**]. These findings demonstrated that full strength MS medium favored the growth and salidroside accumulation in CCA suspension cultures of *R. imbricata.*

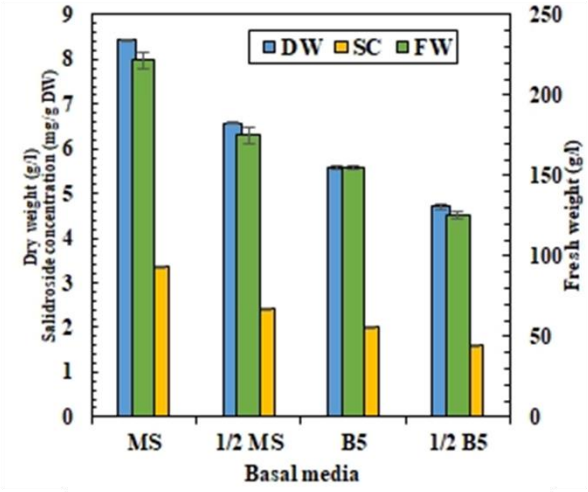

Figure 4.11: Effect of basal media on biomass and salidroside accumulation in CCA suspension cultures of *Rhodiola imbricata.*

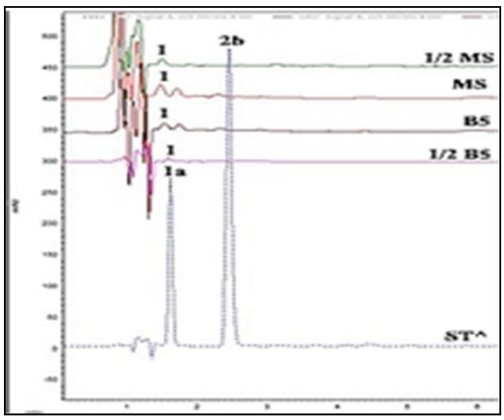

Figure 4.12: HPLC chromatogram illustrating the effect of basal media on phenylethanoids production in CCA suspension cultures of *R. imbricata.* **1a**: Salidroside, **2b**: Tyrosol and **ST**^: Standards.

4.10 Effect of carbon source and sucrose concentration on biomass accumulation and phenylethanoids production in CCA suspension cultures of *Rhodiola imbricata*

Sugar signaling generally regulates growth and secondary metabolites formation in plant cell cultures [**287**]. Since numerous studies have demonstrated the differential effect of different carbon source on growth and bioactive metabolite accumulation in plant cell cultures [**206**], therefore, we need to empirically select a suitable carbon source for optimum response in cell cultures. In this study. the maximum amount of growth (FW: 221.59 ± 0.71 g/l, DW: 8.43 ± 0.01 g/l) and salidroside content (3.37 ± 0.03 mg/g DW) was recorded in MS media containing sucrose (30 g/l), as compared to other carbon sources (**Figure 4.13, Figure 4.14, $p \leq 0.05$).** This differential response could be attributed to varying signaling levels of sugar [**288**]. Different sugars can have different regulatory roles in physiological processes [**289**]. Sucrose generally have long-half life inside the cells, as compared to glucose and fructose, which favours the higher biomass accumulation in plant cell cultures [**289**]. Sucrose is also regarded to be the best carbon source for plant cell cultures due to its efficient intercellular transport, rapid uptake across the plasma membrane and maintenance of optimum osmotic potential [**289**]. The marked influence of sucrose was also previously reported in *in-vitro* cultures of *Withania somnifera* [**288**], *Cistanche deserticola* [**290**] and *Panax ginseng* [**291**]. Liu et al. [**292]** reported that the sucrose induces the biosynthesis of phenolic compounds in apple by up-regulating the expression of the regulatory and structural genes via MdSnRK1.1.

In the present study, sucrose favoured the maximum growth (FW: 221.59 ± 0.71 g/l, DW: 8.43 ± 0.01 g/l) and salidroside accumulation (3.37 ± 0.03 mg/g DW) at 30 g / l sucrose level, but at higher level (50 g / l and 70 g / l) the biomass and salidroside content was adversely affected in CCA culture of *R. imbricata* (**Figure 4.15, Figure 4.16**).These results demonstrated that the higher concentrations of sucrose repressed the growth and salidroside content in CCA cultures, which might be due to substrate inhibition and higher osmotic stress. Zhao et al. [**293**] reported that sucrose concentrations significantly influence the accumulation of alkaloid in CCC cultures of *Catharanthus roseus* by affecting the differentiation level and degree of compaction in CCC cultures. Several studies have suggested that sucrose exceeding 40 g/l brought about a substantial decrease in the average diameter of the cell aggregates, which may result in lower accumulation of secondary metabolites, as it has been postulated that increased cell aggregate

diameter enhanced secondary product formation due to limited diffusion of oxygen in the cell aggregate [**143].** Liu et al. [**295**] found that the 30 g/l sucrose favours growth and echinacoside accumulation in *in-vitro* culture of *Cistanche tubulosa.* The results suggest that 30 g / l sucrose is the most suitable carbon source for growth and salidroside accumulation in CCA suspension cultures of *R. imbricata*.

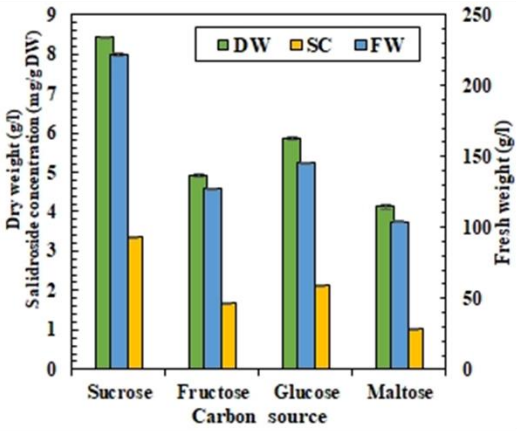

Figure 4.13: Effect of carbon source on Biomass accumulation and salidroside production in CCA suspension cultures of *Rhodiola imbricata.*
Values are mean ± standard error (vertical error bars) of three replicates.

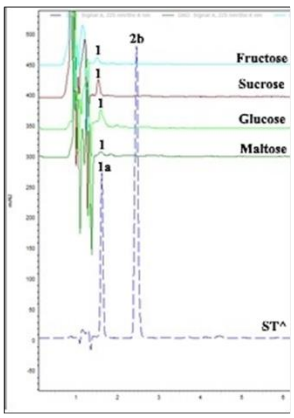

Figure 4.14: HPLC chromatogram demonstrating the influence of carbon source on phenylethanoids production in CCA suspension cultures of *R. imbricata*.
1a: Salidroside, **2b**: Tyrosol and **ST**^: Standards.

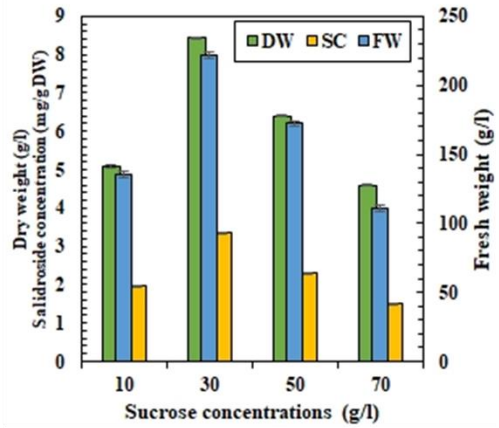

Figure 4.15: Effect of sucrose concentrations on Biomass accumulation and salidroside production in CCA suspension cultures of *Rhodiola imbricata.*
suspension cultures
Values are mean ± standard error (vertical error bars) of three replicates.

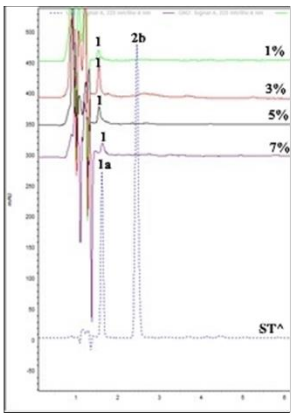

Figure 4.16: HPLC chromatogram illustrating the effect of different concentrations of sucrose on phenylethanoids production in CCA of *R. imbricata*.
1a: Salidroside, **2b**: Tyrosol and **ST**^: Standards.

4.11 Effect of plant growth regulators on biomass accumulation and phenylethanoids production in CCA suspension cultures of *Rhodiola imbricata*

PGRs play a crucial role in regulating the growth and secondary product formation in *in-vitro* cultures [**295**]. However, the response to particular concentrations and combinations of PGRs may vary from plant to plant, therefore, it becomes vital to determine the suitable concentration of PGRs for secondary metabolites production [**296**]. In the present study, 3 mg/l NAA + 3 mg/l BA displayed highest amount of biomass (FW: 221.59 ± 0.71 g/l, DW: 8.43 ± 0.01 g/l) and maximum salidroside content (3.37 ± 0.03 mg/g DW) and salidroside yield (28.45 ± 0.04 mg/l) after 6 days, as compared to other concentrations (**Figure 4.17 a-d, Figure 4.18**, $p \leq 0.05$). NAA and BA at lower concentrations lead to a substantial decrease in salidroside accumulation, as compared to higher concentrations (**Figure 4.17, Figure 4.19,** $p \leq 0.05$). The pH declines during initial 6 days and then rise gradually. The salidroside production was found to be growth-associated, which may be ascribed to relatively differentiated morphology of CCA [**154**]. Similar phenomenon is also reported in suspension cultures of *Saussurea medusa* [**278**], *Cistanche tubulosa* [**294**], *Buddleja cordata* [**297**] and *Scrophularia striata* [**251**]. The optimum combination and concentration of PGRs modulates the expression of the specific biosynthetic enzymes at transcriptional and/or translational level to alter the synthesis of bioactive metabolites [**31**]. Ramawat et al. [**183]** suggests that PGRs stimulates the product formation by probable alterations in cytoplasmatic conditions.

In this study, CCA cultures showed 1.52-fold higher content of salidroside, as compared to the parent plant ($p \leq 0.05$). Shi et al. [**149**] also reported that *in-vitro* cultures of *Rhodiola crenulata* have significantly higher content of salidroside, as compared to the mother plant. The CCA suspension cultures of *R. imbricata* also displayed relatively fast cell growth (1.13-fold) and higher salidroside production (1.24-fold), as compared to callus cultures ($p \leq 0.05$). Similar results were also previously reported in other *in-vitro* cultures [**277**]. The present study highlights that the growth and salidroside production in CCA cultures are remarkably influenced by the concentration of NAA and BA. In the present study, we have successfully optimized the optimum concentration of NAA (3 mg/l) and BA (3 mg/l) for improved synthesis of salidroside in CCA suspension cultures of *R. imbricata*, which is highly desirable from commercial and industrial perspectives.

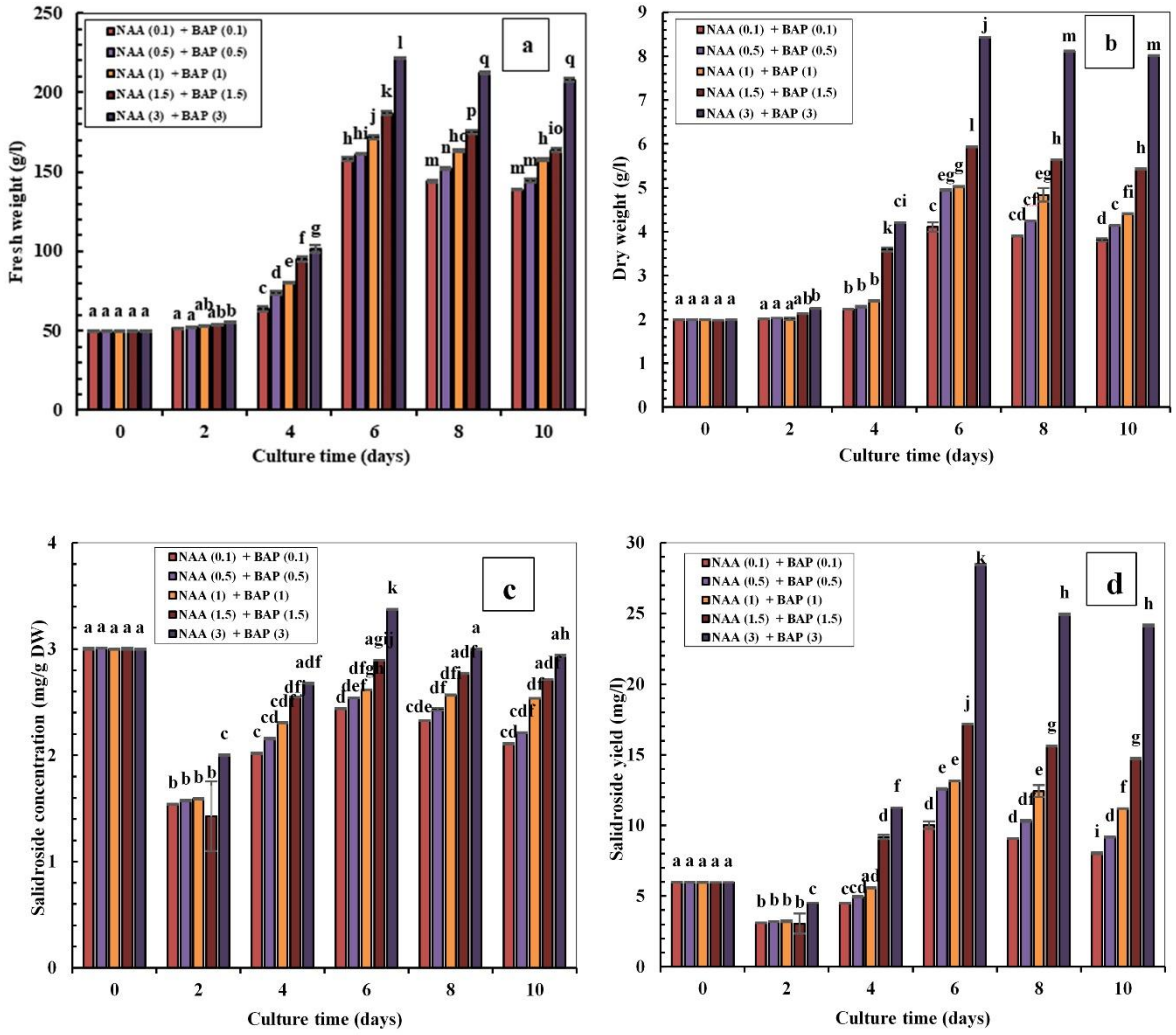

Figure 4.17: Effect of PGRs on (**a**) fresh weight, (**b**) dry weight, (**c**) salidroside production and (**d**) salidroside yield in CCA suspension cultures of *Rhodiola imbricata*. Values are mean ± standard error (vertical error bars) of three replicates. Mean with similar letters are not significantly different at $p \leq 0.05$ according to Bonferroni post-hoc test.

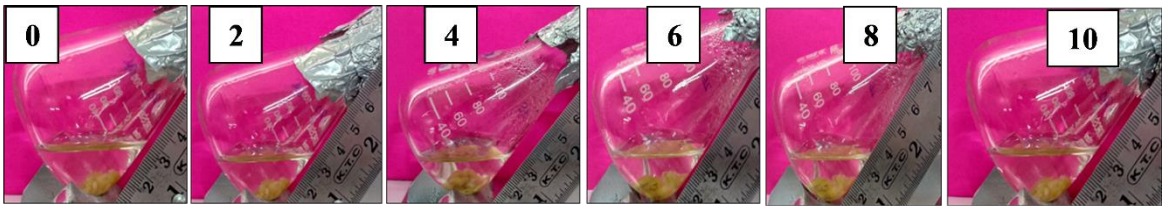

Figure 4.18: Time-course evaluation of biomass accumulation in CCA suspension cultures of *R. imbricata.*

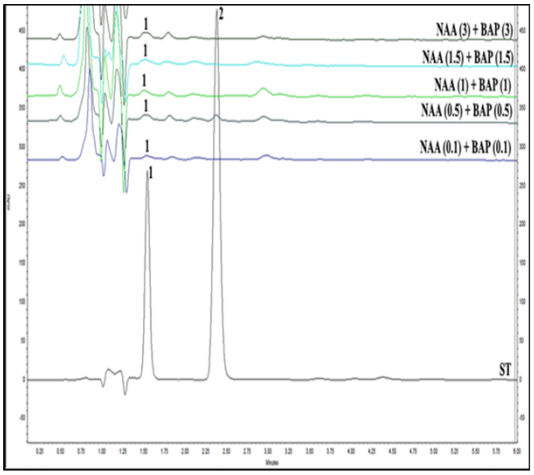

Figure 4.19: HPLC chromatogram illustrating the accumulation of phenylethanoids in CCA suspension cultures of *Rhodiola imbricata* cultivated in MS medium supplemented with different concentrations and combinations of NAA and BAP. **1**: Salidroside, **2**: Tyrosol and **ST**: Standard.

4.12 Effect of light quality on growth in callus cultures of *Rhodiola imbricata*

Light quality modulates plant growth by selective triggering of photoreceptors, including, the triggering of cryptochrome, phytochrome and UV-B receptor by blue, red and far-red light, and ultraviolet light, respectively [**41**]. However, there is still a lack of knowledge regarding the effective light spectrum for improving growth in cell cultures of *R. imbricata.* Therefore, in this study, we investigate the time-course effect of different light conditions on growth in callus cultures of *R. imbricata*. The cultures incubated in different light spectra showed identical extent of growth phases (**Figure 4.20 a**). However, there were significant differences in dry weight, growth rate, doubling time and growth index between different light conditions (**Figure 4.20 c, d, e,** $p \leq 0.05$). Red light treated cultures unveil maximum growth (7.43 ± 0.01 g/l DW) on day 21, as compared to other light treatments (**Figure 4.20 a**, $p \leq 0.05$). The cultures incubated in varied light spectra exhibited heterogeneity in morphological appearance (**Figure 4.20 b)**. The maximum SGR ($\mu = 0.126$ days^{-1}) and minimum DT (t_d =132.66 ± 0.14 hours) was recorded in cultures cultivated under red light, followed by white, blue, RGB and green light (**Figure 4.20 c, d**). The red light treated cultures showed maximum GI (2.97 ± 0.01), as compared to other light treatments (**Figure 4.20 e**, $p \leq 0.05$). It is plausible that red light enhanced the biomass accumulation in callus cultures by increasing the activity and synthesis of growth-related enzymes through physiologically active form of phytochrome (P_{fr}) [**298**]. Moreover, this could also be

attributed to the enhanced photochemical and photosynthetic efficiency [**299**]. The importance of red light for chlorophyll synthesis and chloroplast development is also stated in various studies [**171].** Ouyang et al. [**180**] demonstrated that red light modulates growth in *Picea abies* by regulating the biosynthesis of gibberellins. The present findings could ensure the sustainable supply of medicinally important bioresource in nature-amiable way.

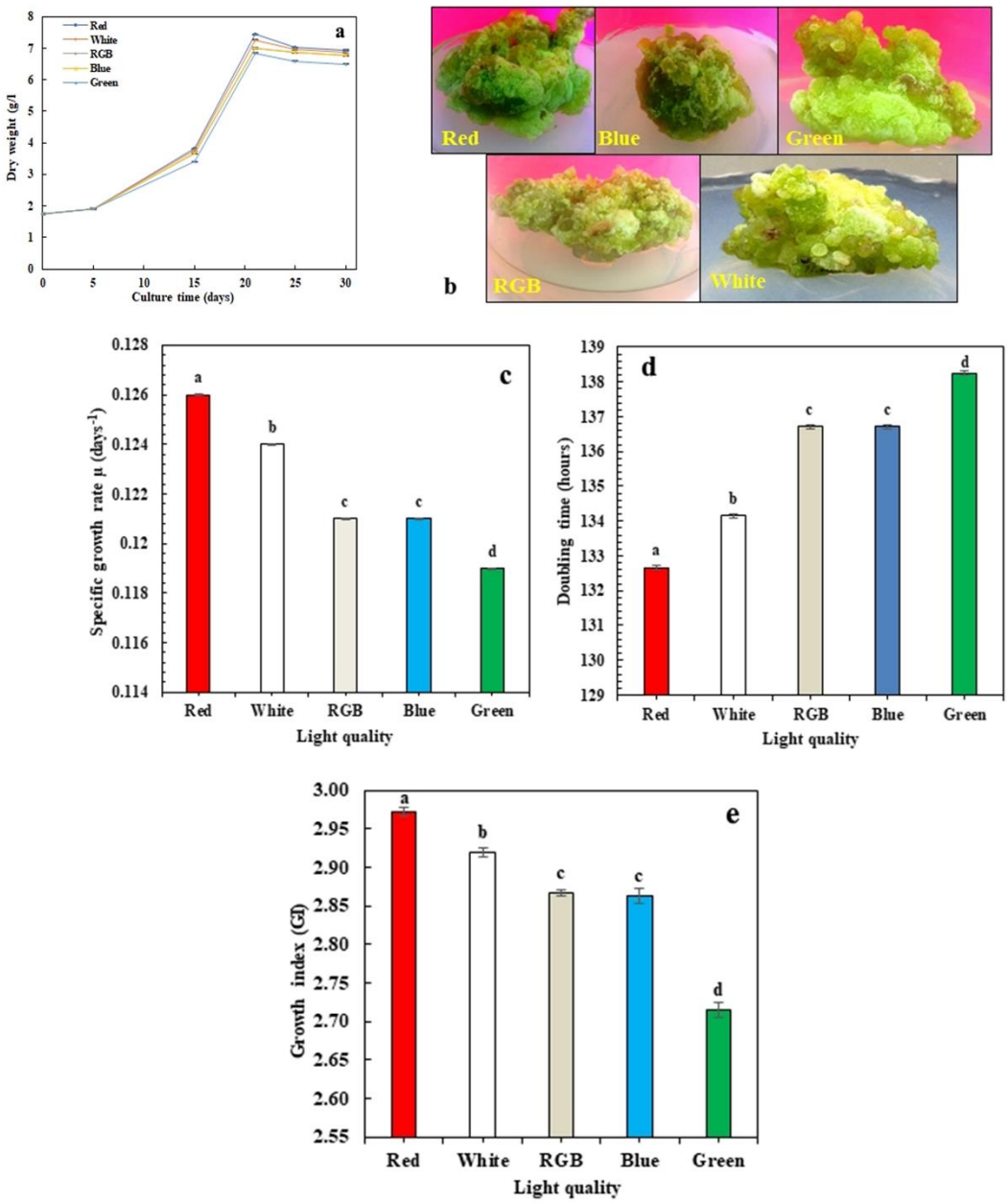

Figure 4.20: Effect of light quality on (**a**) biomass accumulation, (**b**) morphological attributes, (**c**) specific growth rate, (**d**) doubling time and (**e**) growth index in callus cultures of *Rhodiola imbricata.* Values are mean ± standard deviation (vertical error bars) of three replicates. Mean with similar letters are not significantly different at $p \leq 0.05$ according to Bonferroni post-hoc test.

4.13 Effect of light quality on production of phenylethanoids in callus cultures of *Rhodiola imbricata*

Light is regarded as an efficient abiotic elicitor of plant secondary metabolism [**47**]. Light quality regulates several defense-related genes associated with biosynthesis of secondary metabolites in cell cultures [**55**]. In this study, the callus cultures treated with distinct spectral lights showed significant variability in levels of salidroside. However, salidroside was found to be absent in RGB-treated callus cultures. Morevoer, tyrosol was also absent in all the cultures, which warrants further investigation (**Figure 4.21 a**, **Figure 4.22**). The cultures treated with different spectral regimes reveal similar production dynamics (**Figure 4.21 a**). The accumulation of salidroside increased exponentially between day 5 and day 21 and then declined gradually up to day 30. The blue light treated callus cultures showed maximum salidroside content (3.21 ± 0.03 mg/g DW) on day 21, as compared to other light treatments ($p \leq 0.05$, **Figure 4.21 a, Figure 4.22**). The maximum salidroside yield (19.08 mg/l) was recorded in cultures treated with blue light, followed by red, white and green light (**Figure 4.21 b**). The production of salidroside was observed to be growth-associated in callus cultures. These findings for the first time unveil marked impact of blue light on increased buildup of salidroside in cell cultures of *R. imbricata*. This could be ascribed to the higher levels of activated phytochrome (P_{fr}), which regulates the expression of salidroside biosynthetic genes [**300**]. It could also be a protective defense mechanism against high ROS levels [**301**]. Ouyang et al. [**180**] found that blue light stimulates phenolics production in *Picea abies* by upregulating several genes in their biosynthetic pathway. The blue light treatment also increased the accrual of jaceosidin in *in-vitro* cultures of *Saussurea medusa* [**278**]. In the present study, the blue light-treated callus showed 1.45-fold higher content of salidroside, as compared to the parent plant ($p \leq 0.05$). These finding demonstrates the considerable potential of light spectra in regulating the biosynthesis of salidroside in callus cultures. The results unveil that blue light could be efficiently used as an abiotic elicitor for increasing the biotechnological production of salidroside in callus cultures of *R. imbricata*. This elicitation strategy would be of great use for research and industrial applications.

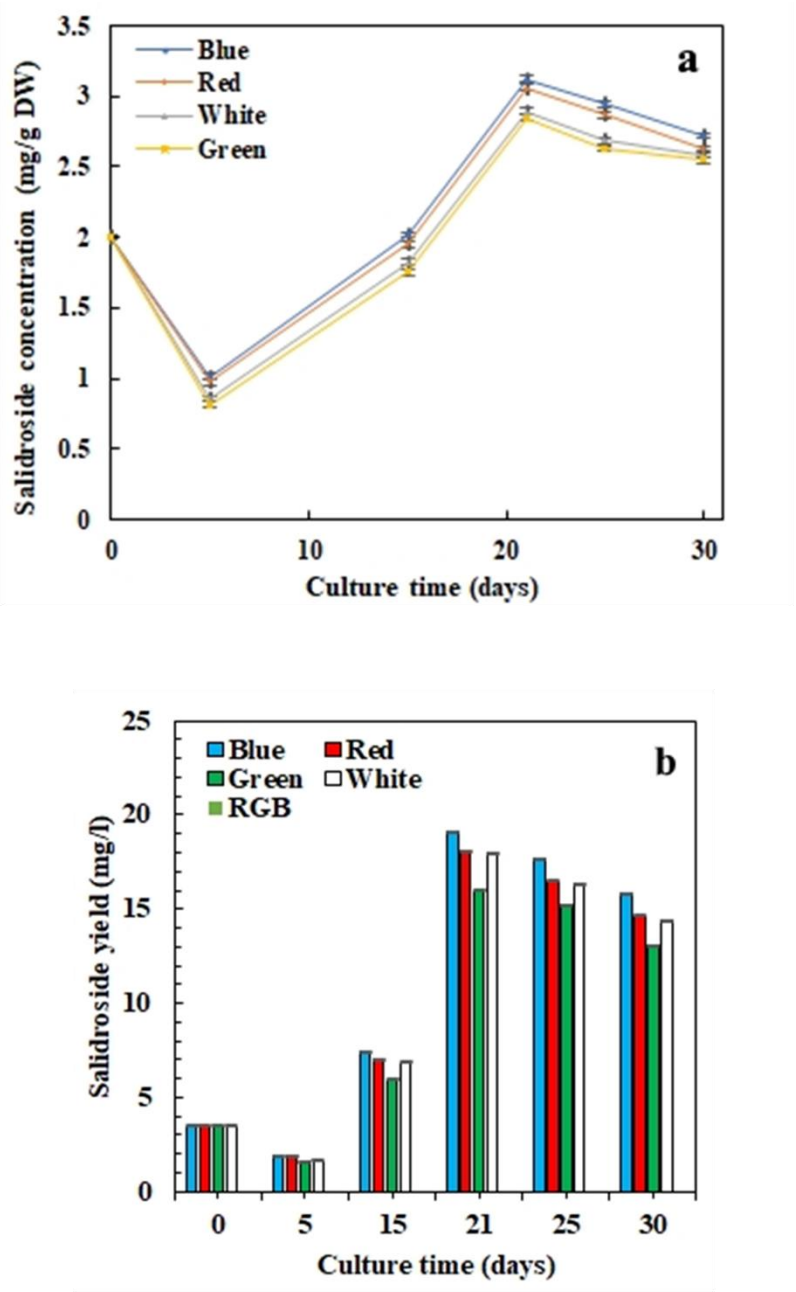

Figure 4.21: Effect of light quality on (**a**) salidroside production and (**b**) salidroside yield in callus cultures of *Rhodiola imbricata.* Values are mean ± standard deviation (vertical error bars) of three replicates.

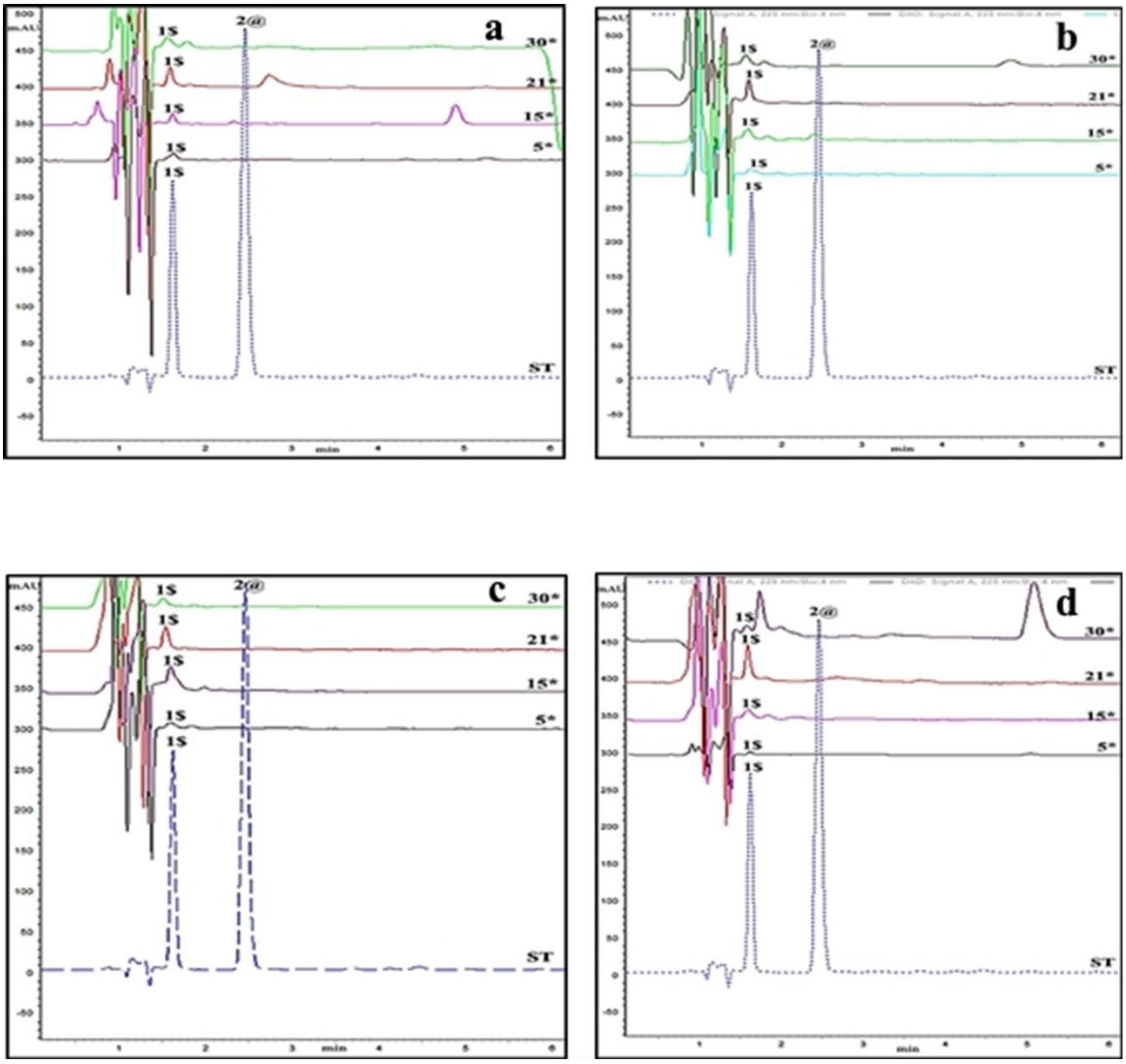

Figure 4.22: HPLC Chromatograms illustrating time-course of Salidroside accumulation in callus cultures of *Rhodiola imbricata* grown under different light conditions. (**a)** Red light, (**b)** Blue light**,** (**c)** Green light and (**d)** White light**. 1$:** Salidroside, **2@:** Tyrosol, **ST:** Standard and * Culture time (days).

4.14 Effect of light quality on total phenolic content and total flavonoid content in callus cultures of *Rhodiola imbricata*

Phenolic compounds have aroused immense industrial interest as antioxidants [**302**]. Several authors have reported the marked impact of light quality on the biosynthesis of phenolics in *in-vitro* cultures [**179**], but, other authors have demonstrated that impact of light quality on synthesis of phenolics is species-specific [**303**]. In this study, the different light-spectra treated callus displayed substantial difference in TFC and TPC. The TPC vary between 6.16 and 11.84 mg CHA/g DW , while TFC vary between 0.53 to 5.53 mg RE/g DW (**Figure 4.23 a**, **Figure 4.23 b**). Blue light treated cultures render maximum TPC (

11.84 ± 1.05 mg CHA/g DW) and TFC (5.53 ± 1.17 mg RE/g DW), as compared to other light treatments (**Figure 4.23 a**, **Figure 4.23 b**, $p \leq 0.05$). These results demonstrate marked influence of blue light on increased synthesis of bioactive phenolics in cell cultures of *R. imbricata*. Several authors have demonstrated that blue light increase the biosynthesis of phenolic compounds by upregulating the expression of PAL, 4CH, CHS, CHI, F3H, FLS-2, UFGT, ANS and MYBA1 genes through cryptochrome and phototropins [**304, 305, 306**]. Several researchers have found that blue light induces accumulation of jasmonic acid, which, in turn, increase synthesis of phenolic compounds [**307].** Blue light can also alter the secondary metabolism for providing protection against abiotic and biotic challenges [**50**]. Several researchers have demonstrated marked bearing of blue light on biosynthesis of phenolics in *in-vitro* cultures of *Saussurea medusa* [**278**], *Scutellaria lateriflora* [**308],** *Schisandra chinensis* [**309]** and *Stevia rebaudiana* [**176**]. These results indicate that this elicitation approach may provide substantial benefits in uplifting the pharmaceutical value of cell culture system. These findings suggest that specific light quality triggers biosynthesis of phenolic compounds in cell cultures of *R. imbricata*. The result highlights for the first time that the application of blue light can enhanced the production of health-promoting and industrially valuable bioactive phenolic compounds in callus culture system of *R. imbricata*.

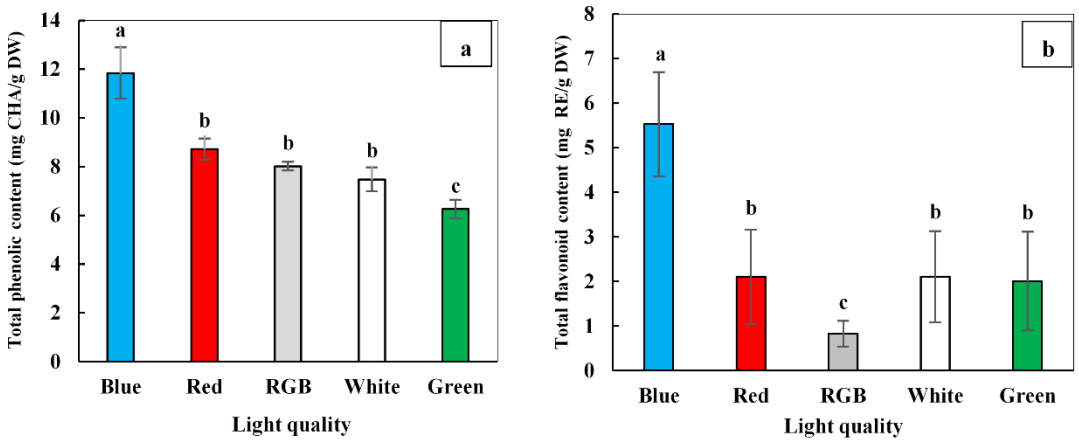

Figure 4.23 Effect of light quality on (**a**) total phenolic content and (**b**) total flavonoid content in callus cultures of *Rhodiola imbricata.* Values are mean ± standard deviation (vertical error bars) of three replicates. Mean with similar letters are not significantly different at $p \leq 0.05$ according to Bonferroni post-hoc test.

4.15 Effect of light quality on ascorbic acid content in callus cultures of *Rhodiola imbricata*

Ascorbic acid acts as a powerful antioxidant and cellular reductant in plants and animals [**310**]. It helps in the prevention of several diseases associated with connective tissue [**311**]. Recent studies have demonstrated that the light can up-regulate the expression of ascorbate biosynthetic and regeneration genes [**247**]. This has inspired us to examine the consequence of distinct spectral lights on the synthesis of ascorbic acid in callus of *R. imbricata*. As shown in **Figure 4.24**, different light spectra treated callus unveil no significant variability in AAC (11.05-13.90 mg/g DW , $p \leq 0.05$). However, several studies have reported differential accumulation of ascorbate under different light conditions [**312**]. Therefore, these responses might be species-specific and warrants further investigation.

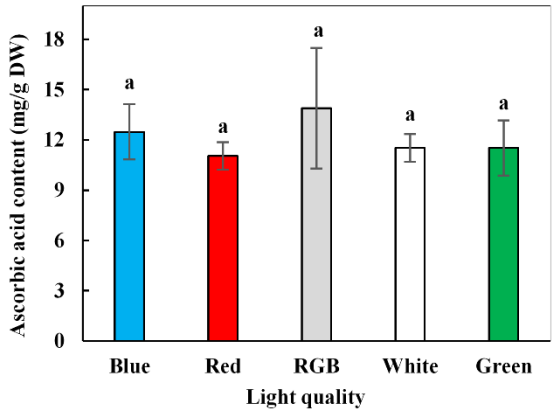

Figure 4.24: Effect of light quality on ascorbic acid content in callus cultures of *Rhodiola imbricata.* Values are mean ± standard deviation (vertical error bars) of three replicates. Mean with similar letters are not significantly different at $p \leq 0.05$ according to Bonferroni post-hoc test.

4.16 Effect of JA on growth in CCA suspension cultures of *Rhodiola imbricata*

The growth of CCA suspension cultures was inhibited by elicitation with JA (**Figure 4.25**). The exogenous application of 5 µM JA markedly repressed the growth (FW: 191.71 ± 0.79 g/l, DW: 7.30 ± 0.01 g/l) in cultures, as compared to control (**Figure 4.25,** $p \leq 0.05$). Furthermore, this effect was further intensified with increasing JA levels (**Figure 4.25**). The supplementation of 100 µM JA resulted in a significant fall in biomass (FW: 177.95 ± 1.51 g/l, DW: 7.05 ± 0.02 g/l), as compared to control ($p \leq 0.05$). These findings reveal that the FW and DW of the CCA cultures of *R. imbricata* fall considerably by higher concentrations of JA. Coste et al. [**313**] also found that higher concentration of JA significantly inhibits the biomass accumulation in shoot cultures of *Hypericum* species. Similar results are also unveiled in *in-vitro* cultures of *Hevea brasiliensis* [**314**] and *Panax ginseng* [**315**]. Ueda et al. [**316**] reported that JA inhibits cell elongation as an antagonist of

IAA. JA also downregulates the expression of the CDK-B, CYCB1;1 and mitotic phase genes that inhibits the cell growth [**317**]. Recently, Ruiz-May et al. [**318**] postulated that methyl jasmonate mediates growth inhibition through perturbations in mitochondrial membrane integrity. Jasmonates treatment also downregulates the expression of histone-encoding genes that led to decrease in cell division in *Taxus* [**319**].

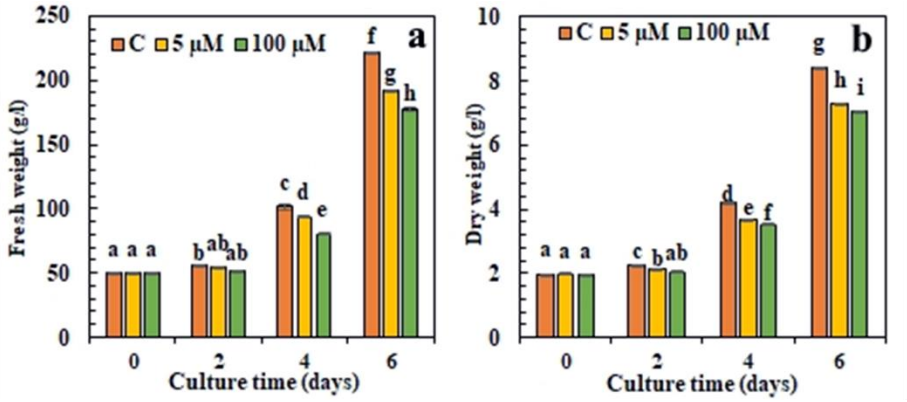

Figure 4.25: Effect of JA on (**a**) fresh weight and (**b**) dry weight accumulation in CCA suspension cultures of *Rhodiola imbricata.* Values are mean ± standard error (vertical error bars) of three replicates. Mean with similar letters are not significantly different at $p \leq 0.05$ according to Bonferroni post-hoc test.

4.17 Effect of JA on production of phenylethanoids in CCA suspension cultures of *Rhodiola imbricata*

Jasmonic acid is involved in the activation of plant defense responses during biotic and abiotic stresses [**320**]. It also stimulates secondary metabolism in *in-vitro* cultures [**58**]. In this study, the highest salidroside content (5.25 ± 0.01 mg/g DW; 1.55-fold higher than control) and salidroside yield (37 ± 0.15 mg/l; 1.34-fold higher than the control) was acquired in 100 µM JA elicited CCA cultures following 4 days of elicitation (**Figure 4.26 a, Figure 4.27,** $p \leq 0.05$). The JA-treated cultures showed 2.36-fold higher content of salidroside, as compared to the parent plant ($p \leq 0.05$). Tyrosol was found to be absent in all the cultures, probably due to their role as precursor for leading to salidroside biosynthesis. These results demonstrated that cell cultures of *R. imbricata* were tractable for JA treatment, as apparent through enhanced production of salidroside. This increase in salidroside biosynthesis is probably mediated via increased transcription of JA-receptive genes. Yu et al. [**321**] reported that exogenously applied jasmonate (250 µM) increased the production of salidrosode in *in-vitro* cultures of *Rhodiola sachalinensis* by upregulating the expression of UDP-

glycosyltransferases (UGTs). It also increased the accrual of phenylethanoid glycosides (echinacoside (21.18 %) and acteoside (5.24 %)) in *in-vitro* culture of *Cistanche tubulosa* [**294**]. Silja et al. [**322**] found that 100 µM JA enhanced the production of plumbagin in embryogenic cell suspension cultures of *Plumbago rosea* after 6 days of culture. Several studies have demonstrated that JA regulates several transcription factors, including AP2/ERFs, MYBs, bHLH and WRKYs, which, in turn, regulates the expression of genes involved in secondary metabolism [**323**]. The present study demonstrates a dose- and time-dependent accumulation of salidroside in CCA suspension cultures of *R. imbricata*. These findings provide a potential biotechnological tool for future commercial production of salidroside under controlled conditions, independent of environmental constrains.

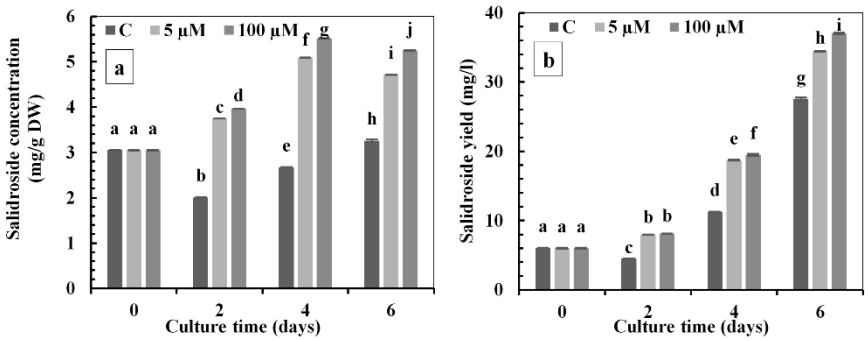

Figure 4.26: Effect of JA on (**a**) salidroside production and (**b**) salidroside yield in CCA suspension cultures of *Rhodiola imbricata.* Values are mean ± standard error (vertical error bars) of three replicates. Mean with similar letters are not significantly different at $p \leq 0.05$ according to Bonferroni post-hoc test.

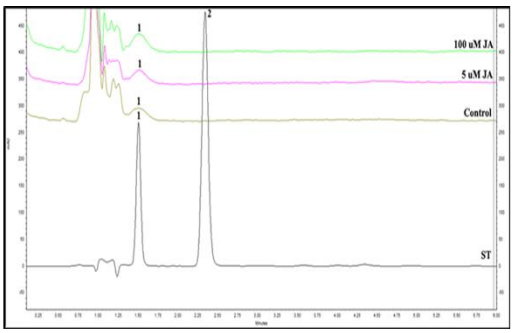

Figure 4.27: HPLC chromatogram illustrating the accumulation of phenylethanoids in CCA suspension cultures of *Rhodiola imbricata* cultivated in Murashige and Skoog (MS) medium supplemented with different concentrations of Jasmonic acid (5 and 100 µM). 1: Salidroside, 2: Tyrosol and ST: Standard.

4.18 Effect of JA on total phenolic content and total flavonoid content in CCA suspension cultures of *Rhodiola imbricata*

Jasmonic acid elicits the accumulation of bioactive compounds in plants, including polyphenols, terpenoids and alkaloids [**324**]. However, the influence of JA on the biosynthesis of phenolic compounds in CCA cultures of *R. imbricata* has not been investigated as of today. In this study, the JA-treated cultures unveil an exponential rise in TFC and TPC following 4 days of elicitation (**Figure 4.28 a, c**). The highest TPC (14.69 ± 0.06 mg CHA/g DW; 1.50-fold higher than control) and TFC (4.95 ± 0.02 mg RE/g DW, 1.68-fold higher than control) was recorded in 100 µM JA elicited culture following 4 days of elicitation (**Figure 4.28 a, c**, $p \leq 0.05$). However, the highest TPP (103.59 ± 0.67 mg/l;1.26-fold higher than control) and TFP (34.89 ± 0.25 mg/l; 1.49-fold higher than control) was recorded in 100 µM JA elicited culture following 6 days of elicitation (**Figure 4.28 b, d,** $p \leq 0.05$). Ali et al. [**204**] also reported that JA (1 mg/l) treatment increased the biosynthesis of TFC and TPC in *in-vitro* cultures of *Artemisia absinthium.* Similar responses were also reported in other *in-vitro* cultures [**324**]. Previous studies have suggested that JA induces the expression of genes encoding key enzymes in the phenylpropanoid pathway (for example, PAL, CHI, CHS and F3H) that leads to the accumulation of bioactive phenolics [**325].** The present study unveils a time- and dose-based response to JA elicitation. Addition of 50 µM of JA significantly improved the levels of flavonoids in cell cultures of *Opuntia megacantha,* but the flavonoids content remained unaltered in *Opuntia ficus-indica* and *Opuntia streptacantha* [**205**]. Similarly, 250 µM JA enhanced hypericin production in shoot cultures of *Hypericum* species, but at different proportions [**313**]. Hence, the response to particular elicitors vary between different plant species, consequently, it becomes pivotal to ascertain ideal concentration of elicitor for product optimization [**326**]. The optimum exposure time of elicitor to plant cell is also an important factor to maximize the elicitation potential [**327**]. Therefore, in this study, we have optimized that 100 µM JA increased the production of TPC and TFC after 4 days of elicitation. The present findings for the first time unveil the feasibility of enhanced biotechnological synthesis of bioactive phenolics from JA elicited CCA of *R. imbricata.*

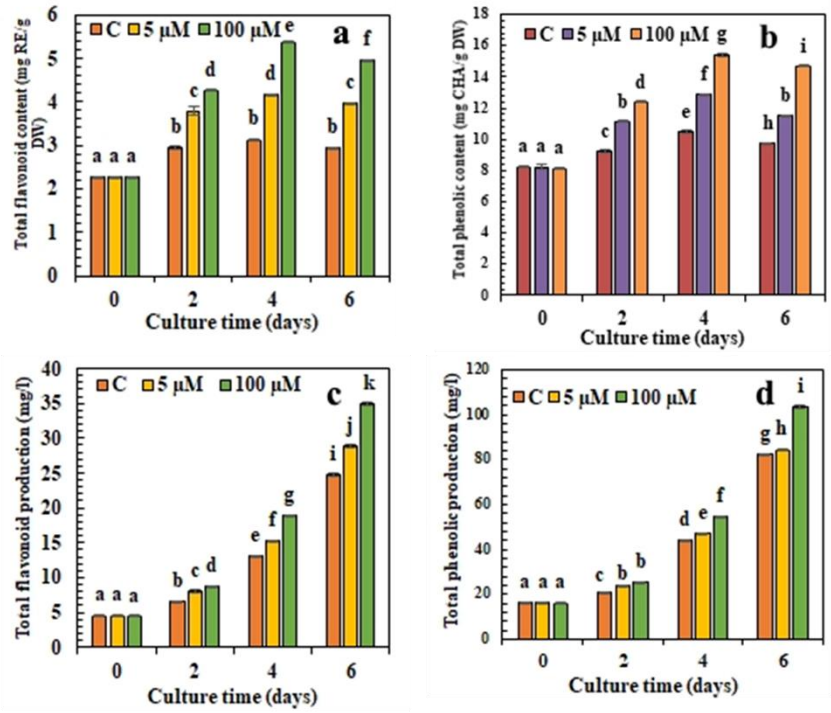

Figure 4.28: Effect of JA on (**a**) TFC, (**b**) TFP, (**c**) TPC and (**d**) TPP in CCA suspension cultures of *Rhodiola imbricata.* Values are mean ± standard error (vertical error bars) of three replicates. Mean with similar letters are not significantly different at $p \leq 0.05$ according to Bonferroni post-hoc test.

4.19 Effect of JA on ascorbic acid content in CCA suspension cultures of *Rhodiola imbricata*

As shown in **Figure 4.29 a**, the JA-treated CCA cultures evince an exponential rise in ascorbic acid content following 4 days of elicitation. The highest AAC (17.93 ± 0.03 mg/g DW; 1.35-fold higher than the control) was recorded in 100 µM JA elicited culture following 4 days of elicitation ($p \leq 0.05$, **Figure 4.29 a**). However, the highest AAP (126.47 ± 0.19 mg/l; 1.13-fold higher than the control) was observed in 100 µM JA elicited culture following 6 days of elicitation ($p \leq 0.05$, **Figure 4.29 b**). Orozco-Cardenas et al. [**328**] observed that jasmonate application spiked the production of ascorbic acid in BY-2 cells of tobacco by inducing the expression of GME and L-GalLDH genes. Nishikawa et al. [**329**] concluded that the jasmonate treatment increased the expression of ascorbate regenerating enzymes (MDAR and DHAR) in broccoli. Shan et al. [**330**] reported that JA up-regulates the ascorbic acid metabolism in maize by activation of MEK1/2 through nitric oxide. The result implies that 100 µM JA-treated CCA of *R. imbricata* could

be expended as a prospective substitute of ascorbate, which have several health-promoting properties [**331**].

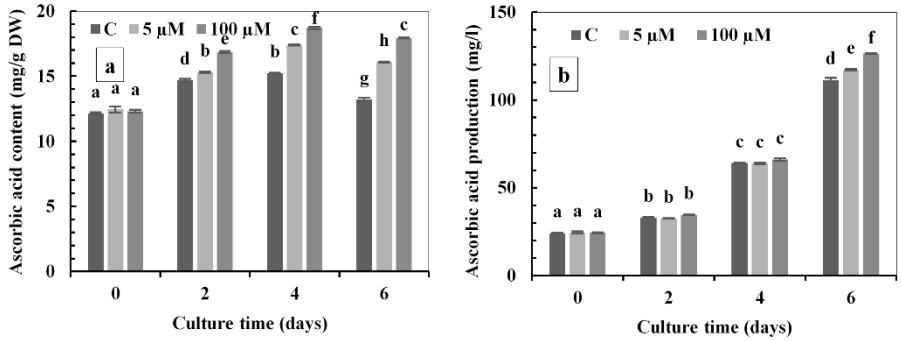

Figure 4.29: Effect of JA on (**a**) ascorbic acid content and (**b**) ascorbic acid production in CCA suspension cultures of *Rhodiola imbricata.* Values are mean ± standard error (vertical error bars) of three replicates. Mean with similar letters are not significantly different at $p \leq 0.05$ according to Bonferroni post-hoc test.

4.20 Antioxidant activity of extracts from callus cultures of *Rhodiola imbricata* chemotypes

Recently, plant-based antioxidants are generating a lot of interest in food industries and human healthcare [**209**]. The antioxidant compounds scavenge excessive ROS for maintaining oxidative stress at endurable levels [**50].** The antioxidant activity of plant extracts can easily be evaluated by several *in-vitro* based assays [**331**]. In present study, we have used DRSA and TAC assay for determining antioxidant activity in callus extracts of different chemotypes (CA1, KA4 and PA5) of *R. imbricata*, because the antioxidant activity cannot be adequately tested using only a single method [**332]**. As shown in **Table 4.10**, the callus culture extracts of KA4 chemotype showed maximum DRSA (49.17 %) and TAC (43.21 mg QE/g DW), as compared to other chemotypes ($p \leq 0.05$). The present study has demonstrated high content of salidroside and non-enzymatic antioxidants in callus cultures of KA4 chemotype, as compared to other chemotypes (**Figure 4.7**). These non-enzymatic antioxidants might have contributed towards the strong antioxidant capacity in callus extracts of KA4 chemotype. Cheng et al. [**277**] reported that the extended conjugation and greater number of hydroxyl groups on the structure of phenylethanoid glycoside might be responsible for substanial antioxidant potential of *Cistanche deserticola* cell cultures. As shown in **Table 4.11**, the Pearson correlation unveil a strong interdependence between antioxidant capacity and ascorbate and phenolics content. Similar interdependence is previously reported in other *in-vitro* cultures [**333**]. The antioxidant property of the phenolics generally depends on the position and number of the hydroxyl moieties [**334**]. The results demonstrated a high

antioxidant potential of callus extracts of KA4 chemotype of *R. imbricata*. These plant-derived natural antioxidants hold great promise for nutraceutical, pharmaceutical and food industries [**32**].

Table 4.10: DFRSA and TAC in callus cultures extracts of *Rhodiola imbricata* chemotypes

	Antioxidant activity	
Chemotypes	**DFRSA (%)**	**TAC (mg QE/g DW)**
CA1	45.14 ± 0.23 [a]	39.37 ± 0.53 a
KA4	49.17 ± 0.29 b	43.21 ± 0.48 b
PA5	42.26 ± 0.28 c	35.55 ± 0.49 c
Standard		
BHA	82.78 ± 0.25 d	293.05 ± 8.58 d
Quercetin	89.00 ± 0.87 e	---
Ascorbic acid	---	408.70 ± 10.15 e

Values are mean ± standard deviation of three replicates. Mean values followed by the different letters within a column are significantly different at $p \leq 0.05$ according to Bonferroni post-hoc test.

Table 4.11: Pearson correlation analysis demonstrating the correlation between the phenolic compounds (SC, TPC, TFC), AAC and antioxidant activity in callus cultures of *Rhodiola imbricata* chemotypes

	DPPH	**TAC**	**SAL**	**TPC**	**TFC**	**AAC**
DPPH	1	.980**	.994**	.908**	.928**	.954**
TAC		1	.993**	.954**	.948**	.967**
SAL			1	.938**	.948**	.959**
TPC				1	.977**	.952**
TFC					1	.960**
AAC						1

** Pearson correlation is significant at $p \leq 0.01$ (2-tailed).

4.21 Antioxidant activity of extracts from light-treated callus cultures of *Rhodiola imbricata*

In the wake of increasing oxidative-stress associated health maladies, the search for natural antioxidants have gained immense importance [**335**]. The natural antioxidants play a vital role in alleviating the detrimental effects of reactive oxygen species [**336**]. In this study, the different light-spectra treated cultures displayed substantial difference in DRSA. The DRSA vary between 43.69 and 53.50 % (**Table 4.12**). The extracts from blue light treated cultures render maximum DRSA (53.50 ± 0.16 %), as compared to other light treatments ($p \leq 0.05$). However, no significant variations were recorded in TAC (27.37 - 30.17 mg QE/g DW, $p \leq 0.05$, **Table 4.12**). Ahmad et al. [**176**] also found a marked influence of blue light on

DRSA in *in-vitro* cultures of *Stevia rebaudiana*. Blue light has been proposed to enhance the antioxidant activity in plants by intensifying the levels of antioxidant compounds, including ascorbate and flavonoids [**303**]. The variability in DRSA and TAC assay might be credited to their diverse mechanism of action [**338**]. The results unveil that extracts from blue light treated culture have maximum antioxidant potential, which could be ascribed as protective mechanism against increased ROS levels [**301**]. The Pearson correlation presented a strong association between TPC and DRSA in different light-treated cultures (**Table 4.13**). These findings are in concurrence with previous reports on other *in-vitro* cultures [**333**]. The phenolic compounds exhibit strong antioxidant activity primarily because of its hydrogen donating capacity, and metal chelating and redox properties [**335**]. These compounds also exhibit indirect antioxidant action by inducing endogenous protective enzymes, and regulating gene expression and signaling pathways [**335**]. These observations suggest that the extracts from blue light treated callus of *R. imbricata* are prospective source of natural antioxidants, which are used as therapeutic agent against cancer, diabetes and neurodegenerative diseases [**335**].

Table 4.12: Effect of light quality on antioxidant activity in callus cultures of *Rhodiola imbricata* after 30 days of culture

		Antioxidant activity	
Light quality		**DFRSA (%)**	**TAC (mg QE/g DW)**
Blue		53.50 ± 0.16 a	29.95 ± 2.47 a
Red		50.42 ± 0.21 b	27.80 ± 2.48 a
Red: Green: Blue		49.60 ± 0.05 c	30.06 ± 0.87 a
White		50.11 ± 0.12 b	30.17 ± 2.12 a
Green		43.69 ± 0.09 d	27.37 ± 1.05 a
Standard			
BHA		86.78 ± 0.05 e	303.74 ± 4.58 b
Quercetin		92.00 ± 0.10 f	---
Ascorbic acid		---	478.30 ± 8.07 c

Values are mean ± standard deviation of three replicates. Mean values followed by the different letters within a column are significantly different at $p \leq 0.05$ according to Bonferroni post-hoc test.

Table 4.13: Pearson correlation coefficient for estimating the correlation between DFRSA, TAC, TPC, TFC and AAC

	DFRSA	TAC	TPC	TFC	AAC
DFRSA	1	0.406	0.846*	-0.003	0.120
TAC		1	0.216	0.314	0.333
TPC			1	-0.092	0.076
TFC				1	0.274
AAC					1

* Pearson correlation is significant at $p \leq 0.01$ (2-tailed).

4.22 Antioxidant activity of extracts from JA-elicited CCA suspension cultures of *Rhodiola imbricata*

Plant-derived natural antioxidants have aroused immense interest for the management of several pathophysiological conditions, including age-related diseases and metabolic disorders [**335**]. Therefore, in this study, we explored the antioxidant activity from extracts of JA-elicited CCA cultures of *R. imbricata*. As shown in **Figure 4.30**, the extracts from JA-treated cultures unveil gradual increase in TAC and DRSA following 4 days of elicitation. The highest DRSA (56.32 ± 0.06 %; 1.04-fold higher than the control) and TAC (60.45 ± 0.31 mg QE/g DW; 1.03-fold higher than the control) was recorded in 100 µM JA elicited culture following 4 days of elicitation ($p \leq 0.05$). Pearson correlation demonstrated strong interdependence of antioxidant activity and ascorbate and phenolics, which is in concurrence with previous reports (**Table 4.14**) [**254**]. The phenolic compounds exert potent antioxidant activity by inhibiting the activity of some of the free radical generating enzymes (lipoxygenases, cyclooxygenase, xanthine oxidase and cytochrome P450 isoforms) [**338**]**.** Moreover, these compounds are excellent hydrogen donors that terminates the cycle of generation of free radicals [**338**]**.** The result implies that 100 µM JA-elicited *R. imbricata* CCA extracts are propitious source of natural antioxidants. The applications of these natural antioxidants are rapidly increasing in cosmetic, beverage and herbal drug industries [**201].**

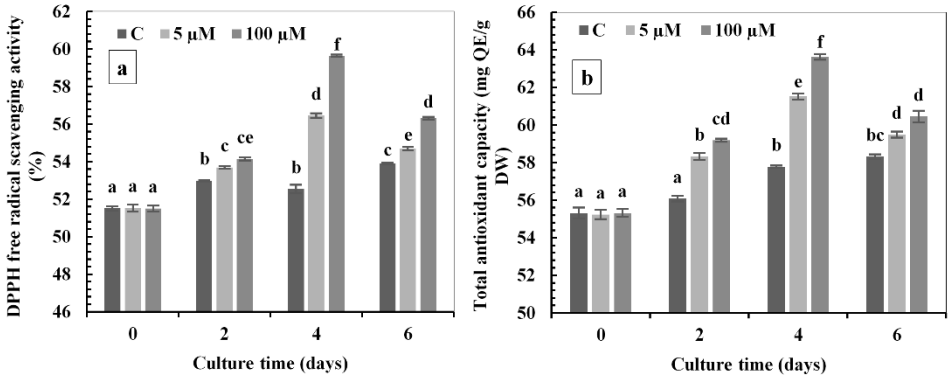

Figure 4.30: Effect of JA on **(a)** DFRSA and **(b)** TAC in CCA suspension cultures of *Rhodiola imbricata.* Values are mean ± standard error (vertical error bars) of three replicates. Mean with similar letters are not significantly different at $p \leq 0.05$ according to Bonferroni post-hoc test.

Table 4.14 Pearson correlation analysis demonstrating the correlation between the phenolic compounds (SC, TPC, TFC), AAC and antioxidant activity in JA-treated CCA suspension cultures of *Rhodiola imbricata*.

	SC	TFC	TPC	AAC	DFRSA	TAC
SC	1	.854**	.870**	.785**	.853**	.875**
TFC		1	.989**	.968**	.924**	.931**
TPC			1	.964**	.935**	.941**
AAC				1	.890**	.909**
DFRSA					1	.962**
TAC						1

** Pearson correlation is significant at $p \leq 0.01$ (2-tailed).

4.23 Antimicrobial activity of extracts from light-treated callus cultures of *Rhodiola imbricata*

The promiscuous use of the conventional antibiotics has substantially contributed towards the increased incidence of antimicrobial resistance [**340, 341].** This, in turn, have resulted in a significant increase in the mortality and morbidity in human populations [**341**]. Therefore, this add urgency to search new pharmaceuticals for the treatment of microbial infections [**340**]. Plant cell culture represents an effective alternative source of antimicrobial compounds [**222**]. In recent years, antimicrobial properties of plant cell culture extracts have been increasingly reported by several researchers [**222**]. Therefore, in this study, the assay demonstrated maximum ZOI (10 ± 1 mm) against *Escherchia coli* in extracts obtained blue light treated callus cultures, as compared to other light treatments (**Figure 4.31 a, Figure 4.32**, $p \leq 0.05$). The extracts from blue light treated cultures exerted maximal antibacterial

action against *Escherichia coli*, with two-fold lower MIC (10 mg/ml) and MBC (20 mg/ml), as compared to other light treatments ($p \leq 0.05$, **Figure 4.31 b, c**). However, no significant activity was observed against *S. aureus* (**Figure 4.31, Figure 4.32**). These differences could be ascribed to variability in composition of cell wall [**211**]. The strong antibacterial action of extracts against *Escherichia coli* might be due to higher amount of flavonoids, as reported in this study (**Figure 4.23**). The flavonoids exhibit strong antimicrobial activity by inhibiting cytoplasmic membrane function, biofilm formation**,** energy metabolism and nucleic acid synthesis in microorganisms [**342**]. Flavonoids also raise bacterial inner membrane permeability, dissipate membrane potential, disrupt proton motive force and inhibit NADH-cytochrome c reductase in the bacterial respiratory electron transport chain [**343**]. Several studies have reported that flavonoids offer protection against *Escherichia coli* by inhibiting the major function of the porin on the cell membrane and restraining the synthesis of peptidoglycan and ribosome [**344**]. Therefore, the extracts from blue light treated callus of *R. imbricata* is a promising source of antimicrobials for pharmaceuticals, alternative medicine and natural therapies.

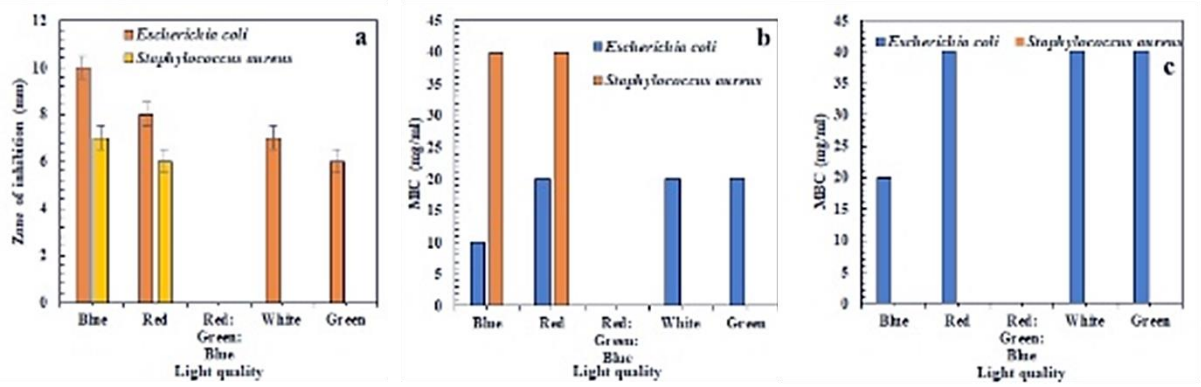

Figure 4.31: Effect of light quality on antimicrobial activity ((**a**) ZOI, (**b**) MIC and (**c**) MBC) in callus cultures of *Rhodiola imbricata*

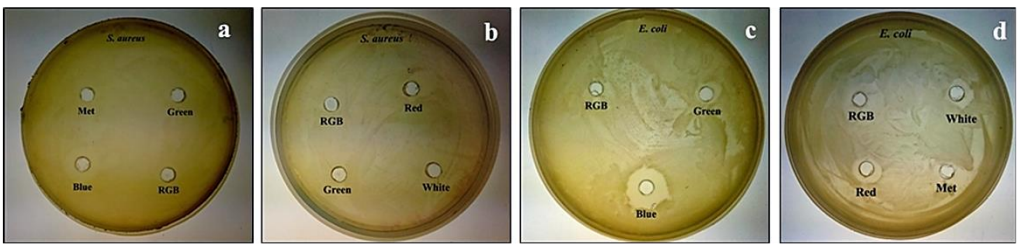

Figure 4.32: Zone of inhibition of different light-treated callus cultures of *R. imbricata* against (**a-b**) *S. aureus* and (**c-d**) *E. coli.*

4.24 Antimicrobial activity of extracts from JA-elicited CCA suspension cultures of *Rhodiola imbricata*

The increasing resistance of pathogenic microorganisms against traditional antibiotics have led to screening of plants cell cultures extracts for bioactive phytochemicals as a potential source of novel antibiotics [**345**]. In this study, the assay showed maximum ZOI (12 ± 1 mm) against *Escherchia coli* from extracts of JA-elicited cultures, as compared to control (**Figure 4.33**, $p \leq 0.05$). The JA-elicited culture exerted maximum antibacterial action against *Escherichia coli,* with two-fold lower MBC (5 mg/ml) and MIC (2.5 mg/ml), as compared to control (**Figure 4.33 b, c,** $p \leq 0.05$). However, no significant activity was observed against *S. aureus* (**Figure 4.33, Figure 4.34**). These differences could be ascribed to variability in composition of cell wall [**211**]. The potent antibacterial action of extracts against *Escherichia coli* might be due to higher levels of phenolics in cultures, as reported in this study (**Figure 4.28**). The phenolic compounds demonstrate potent antimicrobial activity by inhibiting the synthesis of proteins, lipids, polysaccharides and nucleic acid [**346**]. It also inhibits the electron transfer, substrate oxidation, respiratory chain and proton motive force [**346**]. Moreover, it also affects the microbial membrane permeability and membrane functions (electron transport and nutrient uptake) [**347**]. These findings demonstrated that the extracts from JA-treated CCA of *R. imbricata* may serve as a potential antibacterial agent.

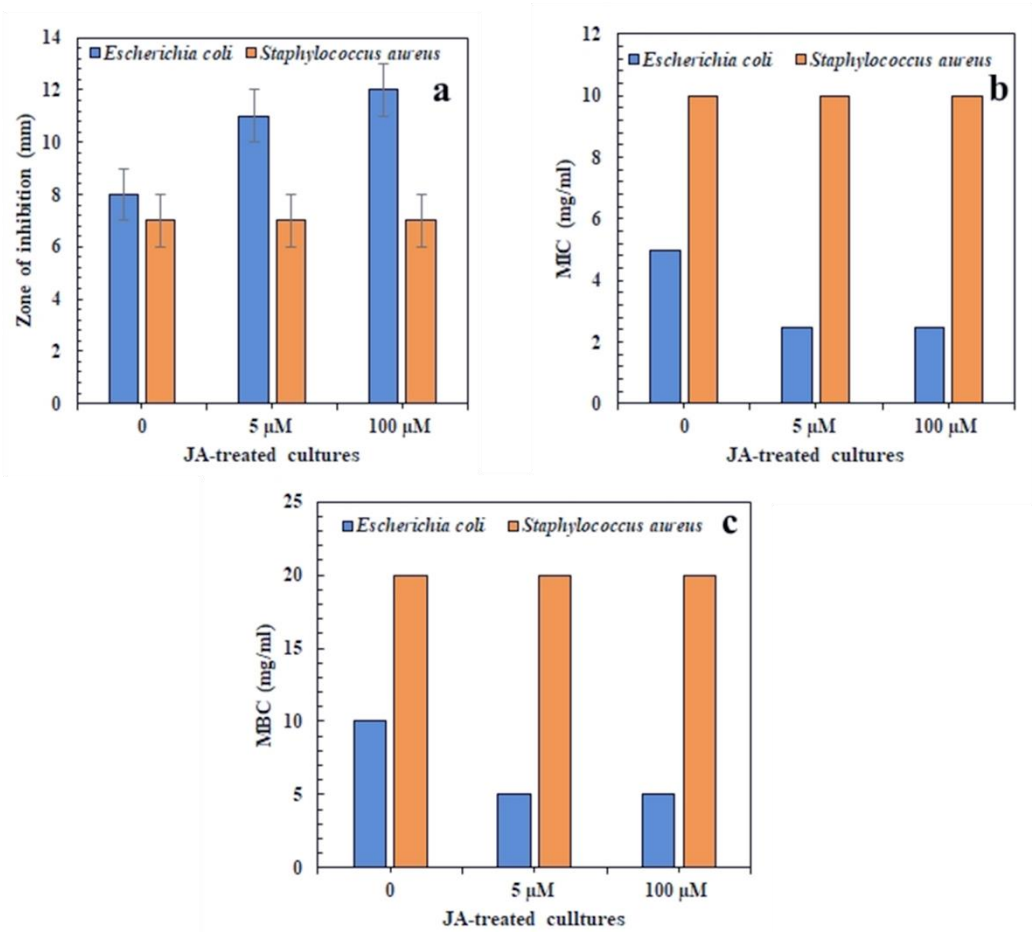

Figure 4.33: Antimicrobial activity ((**a**) ZOI, (**b**) MIC and (**c**) MBC) of JA-treated and untreated CCA suspension cultures of *R. imbricata* against *E. coli* and *S. aureus*.

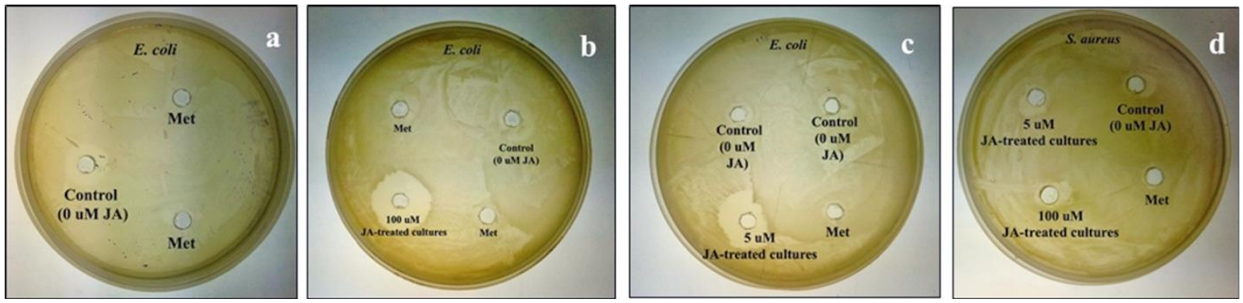

Figure 4.34: Zone of inhibition of JA-treated and untreated CCA suspension cultures of *R. imbricata* against (**a-c**) *E. coli* and (**d**) *S. aureus*.

CONCLUSION AND FUTURE PROSPECTS

Considering the medicinal value, rare status, and lack of information regarding the alternate approaches for the synthesis of bioactive compounds of *Rhodiola imbricata,* the present study unearthed the immense potential of cell culture technologies for sustainable production of pharmaceutically and industrially valuable bioactive compounds of *R. imbricata.* A validated RP-HPLC method for the first time unveil significant variations in content of major phenylethanoids (salidroside and tyrosol) in natural and field-cultivated accessions of *R. imbricata*, which further necessitates the development of effective alternate approaches for consistent production of these major bioactive constituents. KA4 (Khardungla), CA1 (Changla) and PA5 (Penzila) accessions were identified as an elite chemotypes of *R. imbricata* with high content of major bioactive phenylethanoids (salidroside and tyrosol). This study validates the significance of selecting elite chemotypes for obtaining high producing cell lines, as the callus cultures obtained from screened elite chemotype of *R. imbricata* (KA4 (Khardungla accession)) showed high biosynthetic (salidroside, non-enzymatic antioxidants (flavonoids, carotenoids, phenolics, flavonols and ascorbate) and bioactivity (antioxidant activity) potential, as compared to other chemotypes. Rational optimization of cell culture conditions (MS + 30 g/l sucrose + 3 mg/l NAA + 3 mg/l BA) unraveled the untapped biosynthetic (phenylethanoids) and bioactivity (antioxidant activity) potential of cell culture system of *R. imbricata*. The harvesting of compact callus aggregates after 6 days of culture was found to be highly beneficial for obtaining maximum yields of major bioactive constituents. The present study suggested that salidroside production was growth-dependent in cell culture system of *R. imbricata*. GC-MS analysis unveiled the complex chemical profile and therapeutic potential of callus cultures of *R. imbricata,* thereby demonstrating the commercial value and practical utility of cell culture of *R. imbricata.* CCA suspension culture demonstrated higher salidroside (1.52-fold) synthesizing potential, as compared to the parent plant, consequently indicating the promising prospects of cell culture of *R. imbricata* for year-round improved production of medicinally important bioactive metabolites. The blue light demonstrated intense propensity towards the enhanced production of flavonoids (2.62-fold higher than control) whereas red light increased the biomass accumulation (1.10-fold higher than control) in cell cultures. Jasmonic acid (100 μM) treatment was found to be the best elicitation strategy for increasing the production of salidroside (2.37 -fold higher than parent plant), phenolics (1.50-fold higher than control) and

ascorbic acid (1.35-fold higher than control) with concomitant enhancement in biological activities (antioxidant and antimicrobial activity) in cell cultures of *R. imbricata*. Therefore, these findings offer a new feasible biotechnological strategy for enhancing the accumulation of industrially important bioactive compounds in cell culture system of *R. imbricata*. The present study offers novel insights into the morphological and metabolic responses of *R. imbricata* cell cultures towards variable cell culture and elicitation conditions. Since the supply of *R. imbricata* is inadequate, our results bear distinct commercial benefits, as it can comply with escalating desires of industries in an environment-friendly manner. Future studies need to be dedicated on optimization of bioprocess in bioreactors for large-scale production of bioactive molecules from cell culture system of *R. imbricata*.

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LIST OF PUBLICATIONS

Research Publications

[1] **S. Kapoor**, R. Raghuvanshi, P. Bhardwaj, H. Sood, S. Saxena, and O. P. Chaurasia, "Influence of light quality on growth, secondary metabolites production and antioxidant activity in callus culture of *Rhodiola imbricata* Edgew," *Journal of Photochemistry and Photobiology B: Biology*, vol. 183, pp. 258–265, 2018 (IF: 4.067; Scopus & SCI).

[2] **S. Kapoor**, A. Sharma, P. Bhardwaj, H. Sood, S. Saxena, and O. P. Chaurasia, "Enhanced Production of Phenolic Compounds in Compact Callus Aggregate Suspension Cultures of *Rhodiola imbricata* Edgew.," *Applied Biochemistry and Biotechnology*, vol. 187, pp. 817-837, 2019 (IF: 2.277; Scopus and SCI).

National/International Conference Publications

[1] **S. Kapoor**, A. Sharma, H. Sood, S. Saxena, and O. P. Chaurasia, "Phytochemical profiling of callus extracts of *Rhodiola imbricata* by gas chromatography-mass spectrometric technique," *National Seminar on Science & Technology for Sustainable Future*, Rayat Bahra University, Punjab, India, February 27, 2018.

[2] **S. Kapoor**, P. Bhardwaj, R. Kumar, H. Sood, S. Saxena, and O. P. Chaurasia, "Qualitative and Quantitative estimation of phenol glycosides of *Rhodiola imbricata* by RPLC-DAD," *International Conference on advances in plant and microbial biotechnology*, Jaypee Institute of Information Technology, Noida, India, February 2-4, 2017.

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Name of the Supervisor: Dr. HEMANT SOOD

Title of the Thesis/Dissertation/Project Report/Paper (In Capital letters): CELL CULTURE TECHNOLOGIES FOR THE PRODUCTION OF BIOACTIVE COMPOUNDS FROM RHODIOLA IMBRICATA EDGEW. OF TRANS-HIMALAYAN LADAKH REGION

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RECOMMENDATION

Cell culture technologies for the production of bioactive compounds from *Rhodiola imbricata* Edgew. of trans-Himalayan Ladakh region

Considering the medicinal value, rare status, and lack of information regarding the alternate approaches for the synthesis of bioactive compounds of *Rhodiola imbricata*, the present study unearthed the immense potential of cell culture technologies for sustainable production of pharmaceutically and industrially valuable bioactive compounds of *R. imbricata*. A validated RP-HPLC method for the first time unveil significant variations in content of major phenylethanoids (salidroside and tyrosol) in natural and field-cultivated accessions of *R. imbricata*, which further necessitates the development of effective alternate approaches for consistent production of these major bioactive constituents. KA4 (Khardungla), CA1 (Changla) and PA5 (Penzila) accessions were identified as an elite chemotypes of *R. imbricata* with high content of major bioactive phenylethanoids (salidroside and tyrosol). This study validates the significance of selecting elite chemotypes for obtaining high producing cell lines, as the callus cultures obtained from screened elite chemotype of *R. imbricata* (KA4 (Khardungla accession)) showed high biosynthetic (salidroside, non-enzymatic antioxidants (flavonoids, carotenoids, phenolics, flavonols and ascorbate) and bioactivity (antioxidant activity) potential, as compared to other chemotypes. Rational optimization of cell culture conditions (MS + 30 g/l sucrose + 3 mg/l NAA + 3 mg/l BA) unraveled the untapped biosynthetic (phenylethanoids) and bioactivity (antioxidant activity) potential of cell culture system of *R. imbricata*. The harvesting of compact callus aggregates after 6 days of culture was found to be highly beneficial for obtaining maximum yields of major bioactive constituents. The present study suggested that salidroside production was growth-dependent in cell culture system of *R. imbricata*. GC-MS analysis unveiled the complex chemical profile and therapeutic potential of callus cultures of *R. imbricata*, thereby demonstrating the commercial value and practical utility of cell culture of *R. imbricata*. CCA suspension culture demonstrated higher salidroside (1.52-fold) synthesizing potential, as compared to the parent plant, consequently indicating the promising prospects of cell culture of *R. imbricata* for year-round improved production of medicinally important bioactive metabolites. The blue light demonstrated intense propensity towards the enhanced production of flavonoids (2.62-fold higher than control) whereas red light increased the biomass accumulation (1.10-fold higher than control) in cell cultures. Jasmonic acid (100 μM) treatment was found to be the best elicitation strategy for increasing the production of salidroside (2.37 -fold higher than parent plant), phenolics (1.50-fold higher

than control) and ascorbic acid (1.35-fold higher than control) with concomitant enhancement in biological activities (antioxidant and antimicrobial activity) in cell cultures of *R. imbricata*. Therefore, these findings offer a new feasible biotechnological strategy for enhancing the accumulation of industrially important bioactive compounds in cell culture system of *R. imbricata*. The present study offers novel insights into the morphological and metabolic responses of *R. imbricata* cell cultures towards variable cell culture and elicitation conditions. Since the supply of *R. imbricata* is inadequate, our results bear distinct commercial benefits, as it can comply with escalating desires of industries in an environment-friendly manner. Future studies need to be dedicated on optimization of bioprocess in bioreactors for large-scale production of bioactive molecules from cell culture system of *R. imbricata*.